

# **Spermionics: A New Term Describing the Global Survey of the Overall Sperm Function by the Combined Utilization of Immunocytochemistry, Metabolomics, Proteomics and Other Classical Analytical Techniques**

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## **1. Introduction**

Mammalian sperm function is a very complex discipline, involving a great number of functional pathways. This complexity is the result of an extreme specialization in the mechanisms involved in the control of sperm function. We have to remember that sperm is designed with the ultimate role of transmitting male genetic information to the next generation. To this purpose, mammalian sperm firstly undergoes a very complex process of formation, which is initiated at the time of entry to meiosis of the testicular spermatogonia and finished during the epididymal maturation. Subsequently, mature mammalian sperm enter into the female genital tract during the ejaculation. This process initiates a new set of very complicated processes of functional changes, known as capacitation. Capacitation has to be very finely regulated, since there is a close chronological relationship between the time that sperm cells are present in the oviduct and ovulation. This allows sperm cells to reach their optimal functional status at the time of oocyte penetration. However, we must remind that the evolutionary reproductive strategy chosen by each species is different. This has as a direct consequence that the regulatory mechanisms that sperm utilize to modulate their function during their lifespan will be very different among species. These differences will be dependent on the specific schedule of events followed by each species from ejaculation to oocyte fecundation. All of this complexity implies that the experimental procedures that investigators have utilized to know sperm function have shown only a partial picture of it. This includes immunocytochemistry, one of the most important tools in the study of mammalian sperm function. Taking into account these points, it would be necessary to make a brief review of the phenomena during all of these processes in order to get a better comprehension of the techniques by which these phenomena are studied.

### 1.1 A Brief review on sperm formation and its subsequent epididymal maturation

Sperm formation and subsequent epididymal maturation in mammals is a very long and complex process that, in the majority of species, lasts for about two months, with relatively few variations among species (see Clermont, 1972; Wistuba et al., 2007). The formation of a spermatozoon that can display a full ability to reach oocyte fertilization is a process that is commonly divided in three chronological steps; spermatogenesis, spermiogenesis and sperm maturation.

The first step in the mammalian sperm formation is spermatogenesis. This phase is carried out into testes and consists in the formation of haploid germ cells, named spermatocytes, from a basal line of diploid germinal cells named spermatogonia. For this purpose, spermatogonia undergo a meiotic process in which each spermatogonia yield four separate, haploid spermatocytes (Wolgemuth et al., 1995). But meiosis is not only a process in which a diploid cell produces haploid descendants. Meiosis also hosts a process of DNA recombination yielding four haploid cells with distinct, individual changes in their genome (Yauk et al., 2003). The control of spermatogenesis is not well known, basically due to its complexity, although it is well known the control role played by Leydig cells, which acts through secretion of steroid hormones that are introduced inside the tubular stroma through specific androgen binding proteins, as well as the role of apoptosis in the control of spermatocytes formation (Sharpe et al., 1990; Sharpe et al., 1992; Giampietri et al., 2005). All of these processes involve the expression of many separate proteins, as well as many functional changes in these proteins, including variations in their phosphorylation levels (see as example Maekawa et al., 2002) that can be analyzed through proteomics and genomics.

The second step in the mammalian sperm formation is the spermiogenesis. This process is also carried out into the testes. In fact, spermiogenesis is closely linked to spermatogenesis, both being the subsequent step and being underwent in the same placement that spermatogenesis, namely the spermatid tubulae. In the spermiogenesis, the spermatocytes obtained after the meiotic phase undergo a morphological and functional transformation that converts these cells in the direct precursors of spermatozoa (Sharpe, 1994). These precursors, named spermatids, undergo many changes, including the substitution of histones by protamines, the elimination of practically all of the cytoplasm, the transformation of the Golgi apparatus in the acrosome and a pronounced shape change from a rounded one to the typical, elongated shape of the mammalian sperm, including the formation of the tail and the mitochondrial sheath (Tesari et al., 1998). Again, the control of this process is complex and poor understood, although it is well known the key role that Sertoli cells are playing here. Notwithstanding, and in a similar manner than that indicated for the first step of the spermatogenic process, spermiogenesis also involves the expression of many separate proteins, as well as many functional changes in these proteins, including variations in their phosphorylation levels (Hecht, 1995) that can be again analyzed through proteomics and genomics.

The third and final step in the mammalian sperm formation is the epididymal maturation. As its name indicates, this process takes place into the epididymis a long, tubular structure annexed to the testes (Robaire & Hinton, 2002). Spermatozoa formed after the finalization of the spermiogenesis are released from the spermatid tubules to their lumen, which communicates with the epididymis. Afterwards, spermatids travel through the entire epididymis during a time lapse of about fourteen days. During this period, spermatids

undergo several very important modifications which allow these cells to reach their fertilization ability (Jones, 1999). Several of these modifications include total elimination of cytoplasmic remnants in the form of cytoplasmic droplets, final condensation of sperm nucleus and release of sperm proteins to the epididymal lumen, the phenomenon that is concomitant with the uptake of extracellular, epididymal proteins by sperm (Lasserre et al., 2001). As in the other phases of sperm formation, the regulation of all of these changes is not well known and, again, the genomic and proteomic techniques will be basic to the complete understanding of this process.

## **1.2 Changes in sperm function after ejaculation: capacitation**

The final result of all of the above described processes is a mature spermatozoon, which is stored into the final zone of the epididymis until ejaculation or its destruction and subsequent reabsorption. However, the mature spermatozoon that is stored in the final segment of the epididymis lacks neither motility nor a real fertilizing ability in mammals (Vijayaraghavan et al., 1996). These properties are only reached after ejaculation. In this way, motility is stimulated after the contact of spermatozoa with seminal plasma and female genital tract fluids during and immediately after ejaculation, through several signaling compounds that are found by sperm cells after ejaculation such as prostaglandins, estrogens or even neurotransmitters like dopamine (Lindholmer, 1974). On the other hand, the fully fertilizing ability of mammalian spermatozoa is only reached after undergoing a progressive process named capacitation. The capacitation is progressively undertaken during the journey of spermatozoa through the entire female genital tract. In this way, spermatozoa that reached oviduct and waiting there the pass of the ovulated oocytes have undergone several considerable functional changes that allow them to be able for oocyte penetration (Chang, 1984). In the last years there has been a considerable amount of literature devoted to this point and capacitation implies important changes in sperm function such as a complete transformation of the cell membrane fluidity, activation of acrosome components, changes in the tyrosine phosphorylation and hence in the protein activity, of a great array of sperm proteins, displacements of calcium intracellular fluxes or the adoption of an specific motility pattern named hyperactivated motility (Bedford, 1983; Yanagimachi, 1994a; Visconti et al., 1998). Notwithstanding, although these changes are well described in many mammalian species, there are many unknown aspects regarding the regulation of sperm capacitation yet and, at this moment, this is one of the most studied aspects of sperm function.

Thus, as this brief summary has tried to highlight, mammalian sperm physiology is a very long and complex process, not fully studied and understood in any means. The importance of the study of these processes is very great if we want to optimize the strategies by which we can optimize the reproductive indexes in all of the mammalian species, including man. For this purpose, the utilization of tools like genomics and proteomics, specially adapted to the peculiar characteristics of sperm function will be basic in the understanding of the overall sperm function.

## **2. Common instrumental approaches to the study of mammalian sperm function**

Until now, mammalian sperm function has been studied by using techniques that are focused in individual and very particular aspects of this overall function. As an example, the

achievement of capacitation has been linked to a myriad of processes, such as increase in overall protein tyrosine phosphorylation, structural changes of cell membrane, activation of protein kinase A or processing of proacrosin to acrosin (Bedford, 1983; Langlais & Roberts, 1985; Yanagimachi, 1994a; Visconti et al., 1998; Lefièvre et al., 2005). These studies have been carried out separately, and the global interpretation of data can be inferred only after a long and often arduous process of data integration and comparison of results among the published literature. Additionally, if we consider the considerable differences among species, the interpretation of all the collected data are even more difficult, delaying thus a comprehensible interpretation of the overall mammalian sperm function. Despite these problems, the utilized techniques have yielded a great deal of useful information, which has allowed investigators to have a reasonable knowledge of the overall sperm function. Basically, the common instrumental approaches utilized until now here have been of three separate types, namely enzymatic analyses, determination of metabolite levels and detection of protein expression and location.

## 2.1 Enzymatic analyses

This chapter is not devoted to the detailed description of these techniques, although they are important, since enzymatic analyses allow investigators to determine the exact, precise activity of an individual enzyme in a specific point of the sperm lifespan, evaluating thus putative variations of these activities during the entire life of the cell. Concomitantly, the enzymatic analyses also allow investigators to determine the catalytic properties of a specific sperm enzyme, aiding thus to the identification of the exact isozyme that is present in the sperm. In this way, enzymatic analyses are important in order to reflect sperm function in a particular situation.

There are many examples of determination of enzymatic activities in sperm ejaculates, from regulatory enzymes of sperm energy metabolism, like hexokinases (Medrano et al., 2006a), to proteins related to acrosome activity, like acrosin (Cui et al., 2000). In all cases, these techniques are all based in spectrophotometric techniques, in which the specific activity of the studied enzyme is utilized to form (or destroy) a final substrate with optical activity, such as NAD<sup>+</sup> (Passoneau & Lowry, 1993). This serves to detect the rhythm of variation of this optically active substrate during a controlled time lapse in standard conditions of total volume and temperature. After this, the specific activity of the studied proteins can be expressed as specific activities of the analyzed enzymes or results can be utilized to determine the enzyme kinetics by using of diagrams such as the Lineweaver/Burke representation, after determination of enzyme activity in the presence of increasing concentrations of the specific substrate (see Fernández-Novell et al., 2006 as an example of both expressions of hexokinase activity in boar sperm). Although these techniques have been also widely utilized in any tissue of cell sample, the determination of enzymatic activities in mammalian sperm can present a specific variant that is almost uniquely developed for these cells. This variant is due to the very high proportion of non-soluble structures together with the extraordinarily low amount of cytoplasm that presents mammalian sperm when compared to other eukaryotic cells. This peculiarity implies that many sperm proteins are distributed among the soluble and the no-soluble fraction of sperm extracts after their homogenization and centrifugation under following standard protocols (see Medrano et al., 2006a; Medrano et al, 2006b as examples). This distribution and its putative changes following overall changes in the sperm function can contribute in a

relevant form in the control of the specific activities of these enzymes, being thus an additional mechanism of control of the enzyme activity that is almost specific for sperm. In this way, determination of a specific enzymatic activity in mammalian sperm would not be uniquely directed towards the analysis of supernatants obtained after homogenization/centrifugation of samples, but also towards the corresponding pellets, that would have resuspended in known quantities of the corresponding homogenization buffer (see Fernández-Novell et al., 2004 as an example of the analysis in both supernatants and pellets of homogenized samples of hexokinase activity in boar sperm).

## 2.2 Metabolic analyses: metabolite levels

The control of the energy levels is a very important point in order to maintain sperm function during their complete time life. This control is not only regulated by changes in the activity of position of the implied enzymes, but also through variations in the intracellular levels of metabolites involved in this process, such as ATP (Hammersted & Lardy, 1983). As in the case of the techniques devoted to the analysis of enzymatic activities, this chapter is not devoted to describe the techniques developed to determine intracellular levels of metabolites, and they are described elsewhere in the literature. Summarizing, the great majority of metabolite analysis are based in spectrophotometric techniques, similar to that developed for evaluating enzyme activities. However, in case of metabolites, the developed techniques are more frequently developed in an endpoint basis, in which all of the metabolite present in the sample is completely degraded by the addition of the corresponding, specific enzymes and other substrates. This leads to the obtainment of the maximal levels of optically active derived substance obtained from the degradation of the studied metabolite. Treatment of samples usually required as a first step the deproteinization of samples. This is done either through acid precipitation with substances as perchloric acid, as for determining ATP (Lambrecht & Transtschold, 1984) or basic precipitation with substances like sodium hydroxide, as for determining fructose 2,6-bisphosphate (Gómez-Foix et al., 1991). This implies that all of the protein content of sperm samples will be precipitated and, in these conditions, is not possible to evaluate a putative fractioning of metabolites between the soluble and the no-soluble fraction of sperm homogenates treated as per the determination of enzyme activities. In case of polymeric metabolites, such as glycogen, samples must be pretreated in order to degrade the polymer into their monomers by using the appropriate degrading enzymes. For instance, determination of intracellular sperm glycogen levels requires a previous incubation of  $\alpha$ -amylglucosydase, in order to release all of the glucose contained in the polymer (Ballester et al., 2000; Palomo et al., 2003). Then, this released glucose is determined through a standard, spectrophotometric technique (Ballester et al., 2000; Palomo et al., 2003).

## 2.3 Protein expression analysis: Western blot

The determination of the catalytic activity of an enzyme is not the unique form to study the functional role of this protein. In fact, there are many proteins that have not enzymatic properties. In this manner, these no-enzyme proteins can not be studied through techniques involving enzymatic analyses. Thus, another valid approximation to determine the role of a specific protein in the overall sperm function is to evaluate other characteristics that can control its activity. The two most important mechanisms involving control of protein activity are modulating both the total protein content and the phosphorylation levels on

tyrosine, serine and threonine residues (Isen et al., 2006). This is especially important in mature mammalian spermatozoa, since these cells can not control the total content of a protein through modulation of its gene expression (Watt & Coffey, 1991). In this manner, molecular biology techniques involving studies of gene expression are not relevant in spermatozoa. This leads to that the most important techniques to determine both total content and phosphorylation levels of a sperm protein involved the utilization of specific antibodies. The most common of the techniques utilized to determine both the total content and phosphorylation levels of a specific protein is the Western blotting of transferred samples after being subjected to an electrophoresis onto an SDS-polyacrylamide support (SDS-PAGE). Again, this chapter is not devoted to the description of this technique, although it is necessary to indicate that Western blotting is currently one of the most widely utilized techniques in the study of mammalian sperm function, existing a very high amount of articles published in which this technique has been utilized to analyze proteins like hexose transporters (Rigau et al., 2002; Sancho et al., 2007), protein kinases (Vijayaraghavan et al., 1997; Breitbart & Naor, 1999) or even nucleoproteins (Flores et al., 2008; Flores et al., 2011). The usefulness of Western blotting is evident, although it is a technique that can cause many troubles to the unwary. One of the most important troubles that can be present in this technique is the lack of specificity of the antibody utilized to detect the studied protein. This is a problem that can have no easy solution, and, in this way, Western blotting requires in an unavoidable manner the presence of the adequate negative and positive controls in order to assure that the detected bands corresponds without doubts with the studied protein.

#### **2.4 Protein location analysis: immunocytochemistry**

Sperm protein function can be also studied through another technique involving the utilization of specific antibodies. In this way, sperm proteins can modify their activity also through changes in their specific location inside the sperm structure. These location changes can be studied through immunocytochemistry, which detect not only these changes but also other aspects like variations in the intensity of protein phosphorylation following functional changes (see 41 as an example). The immunocytochemistry has been also a widely utilized technique, which can be described in a very great number of articles devoted to sperm function (see Baccetti et al., 1988; Sutovsky et al., 2001; Albarraçín et al., 2004 as examples). However, and in a similar for to that indicated for the Western blotting, the immunocytochemistry is not a totally straightforward technique, existing the possibility to fall into mistakes with a non easy resolution. In this way, troubles related with the specific fixative technique utilized can be of importance. For example, fixation of samples with ethanol rendered a different location of several hexose transporters in boar sperm when compared with similar samples fixed with paraformaldehyde (Rigau et al, 2002; Sancho et al., 2007; Bucci et al., 2010). In this way, there are several applications of immunocytochemistry that are completely valid for spermatozoa. In this manner, one of the most utilized is that centered in the location of submembrane proteins, like hexose transporters or proteins linked to other sperm structures, such as peri-mitochondrial actin and mitofusin-2. The most feasible immunocytochemistry of these proteins is carried out as follows:

Spermatozoa are washed three times with PBS. After the third washing, samples are centrifuged at 600 g for 10 min at 25°C, and the subsequent cellular pellet is resuspended in

a 4% (w/v) paraformaldehyde solution in PBS for 15 min at 25°C. Fixed cells are centrifuged again at 600 g for 3 min at 25°C, and the supernatants are discarded. The cellular pellet is resuspended in 500  $\mu$ L of PBS. This cell suspension is subsequently seeded into gelatin-coated slides of 76 mm x 26 mm of surface. Slides are afterwards covered with a 0.2% (v/v) Triton X-100 solution in PBS for 30 min. This step is absolutely necessary in spermatozoa, since the structure of these does not allow antibodies for an easy entering into the cell. Thus, spermatozoa need to be permeabilized in order to allow antibodies for their penetration inside the sperm cell structure. Slides are then washing with PBS and further incubated in a blocking solution containing 1% (w/v) bovine serum albumin (BSA) for 30 min at 4°C. After a new washing with PBS, slides are then incubated at 4°C with the appropriated primary antibody solution. The dilution and the incubation time will be variable, depending on the specific, utilized antibody. Notwithstanding, as a general rule, the incubation with the majority of the utilized primary antibodies will be carried out overnight, with an antibody dilution in the range of 1/100-to-1/500 (v/v) in PBS. After the incubation with the primary antibody, the slides are washed again with PBS and subsequent incubated with the appropriate, fluorochrome-conjugated secondary antibody. Again, the dilution and the incubation time will be dependent on the specific secondary antibody that will be utilized, although as a general rule a time incubation of 1h is enough. Moreover, the secondary antibody solution is mixed with another solution of the nuclear stain Hoechst 33258 at a final concentration of 1  $\mu$ g/mL. The addition of the nuclear staining is needed in order to perform and adequate identification of the whole sperm structure, facilitating thus the precise location of the obtained markings. Afterwards, slides are thoroughly washed for 4 times with PBS and mounted in the DABCO solution. This solution is composed by 50% (v/v) glycerol and 25 mg/mL 1,4 diazabicyclo [2,2,2] octane in water. Mounted slides are stored at 4°C in the dark. In these storing conditions, fluorescence can be optimally maintained for a maximum of 15 days. Figure 1 shows the final results obtained with this technique, after the observation of samples through a laser confocal microscope.

An interesting variation of the fixation technique consists in the application of cryoconservation techniques. This technique is specially indicated in the study of nuclear sperm proteins, which are difficult to detect in whole cells. Effectively, mature spermatozoa are cells with a very low volume and this study is usually carried out in whole cells. This is not a problem for the great majority of sperm proteins, since the small volume of sperm allows antibodies for a good penetration into the cell after its detergent-caused permeabilization. However, there are proteins that are not accessible for antibodies in whole spermatozoa. The most important of these proteins are those linked to the DNA in the nuclear structures, such as protamines and histones. The only manner in which antibodies against these proteins can contact with their specific proteins is after sectioning of sperm. However, this is practically impossible in samples treated with a standard fixation/inclusion procedure, since the width of the slice that investigators can obtain render a very small percentage of good sperm sections. Cryofixation and further cryosection allows investigators to obtain better results, as shown in (Flores et al., 2008; Flores et al., 2011) and Figure 2, and the procedure is the following: The procedure will start by the washing of sperm samples three times with PBS and subsequent fixation with 500  $\mu$ L of a 2% (w/v) paraformaldehyde solution in PBS for 15 min at 25°C. Fixed samples will be centrifuged at 600 g for 3 minutes, and the supernatants will be discarded. The cellular pellet

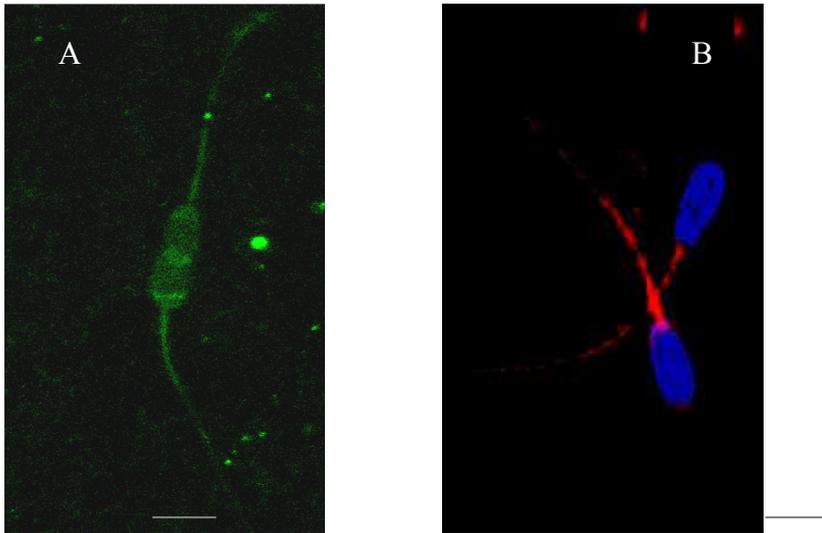


Fig. 1. Immunocytochemistry of actin (A) and mitofusin-2 (B) performed in boar spermatozoa. Figures show the location of both proteins after the utilization of an Alexa 488-conjugated donkey anti-goat secondary antibody for actin and an Alexa 647-conjugated goat anti-rabbit secondary antibody for mitofusin-2. The nuclear stain with Hoechst 33258 is also evident in (B). Bars indicate a real size of 7  $\mu\text{m}$ . Figures are taken from photographs made for the work published in Flores et al. (2010).

will be then resuspended in 500  $\mu\text{L}$  of PBS and centrifuged again at 600  $g$  for 3 min. Supernatants will be again discarded, and the pellets obtained will be embedded in 40  $\mu\text{L}$  of the any cryo-inclusion medium, like the OCT1 (Leica Instruments; Wetzlar, Germany). Samples will be immediately frozen with liquid  $\text{N}_2$  and stored until their processing at  $-80^\circ\text{C}$ . When stated, the included samples will be sectioned in slices of 1 mm of thickness by using a cryostat. Sections will be subsequently placed onto gelatin-coated slides (76 mm x 26 mm). Immediately, the slides will be covered with a PBS solution containing 0.1 (v/v) commercial Hoechst 33258 solution (Boehringer Mannheim). This stain will allow for the determination of an exact co-localization between the signal obtained with the specific antibody and the sperm nuclear DNA in case of the study of the interaction between DNA and a specific nuclear protein. Incubation with Hoechst 33258 will be maintained for 15 min at  $38.5^\circ\text{C}$ , preventing any light source from reaching the slides. Afterwards, samples will follow the standard immunocytochemistry procedure.

Although fixation can be a serious trouble in the immunocytochemistry procedure, it is not the only one that can be found. In fact, the worst troubles could be linked with the specificity of the antibodies utilized to detect a protein. This trouble, that is similar to that described for the Western blotting, can cause many problems of identification and interpretation and, similarly to that indicated for Western blotting, immunocytochemistry requires in an unavoidable manner the presence of the adequate negative and positive controls in order to assure that the detected marks correspond without doubts with the studied protein.

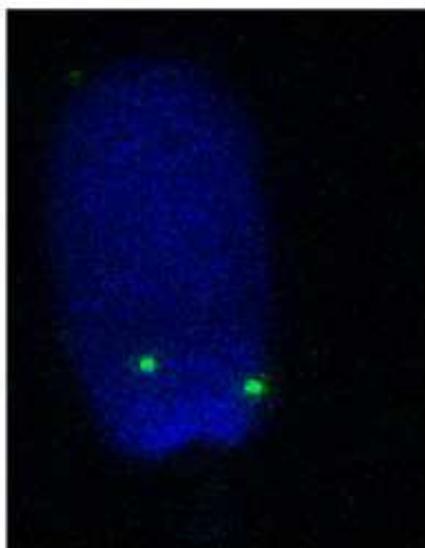


Fig. 2. Detection of histone H1 in the head of freshly obtained boar spermatozoa following the cryofixation and further cryosection technique. Green spots show the location of histone H1 in the head of boar sperm. Nuclear staining has been carried out after the utilization of the Hoechst 33258 stain. The image has been taken from Flores et al. (2011).

### **3. Morphological and functional characteristics that mediates application of technical global approaches in mammalian sperm**

As described above, the study of mammalian sperm has been carried out by applying analytical techniques that are common to all of the other cell types. However, mammalian sperm is a very specific cell, with many typical characteristics that difficult not only the application of the classical techniques, but also the interpretation of the obtained results. Taking into account this, a succinct description of these characteristics is needed in order to a correct interpretation of the new techniques applied to the study of mammalian sperm biology. For this purpose, we have classified these sperm particularities in morphological aspects and functional characteristics.

#### **3.1 Morphological characteristics of mammalian sperm**

The mammalian sperm is a cell with a very characteristic shape, which can not be confused with any other cell. This is due to the fact that these cells are specifically designed to reach their ultimate goal, the penetration of an oocyte. This characteristic morphology also implies that the whole cellular structure of sperm is totally different to the other cells. These differences include aspects such as the practical absence of cytoplasm and cytoplasmic organelles like the Golgi apparatus, ribosomes, lysosomes or endoplasmic reticulum, the presence of a haploid, highly compacted nucleus and the existence of specialized organelles that are specialized products from classical cell structures. In this last group the most important are the acrosome, which is a derived of the Golgi apparatus, and the

mitochondrial sheath, which is a specialized structure formed with very tightly bound mitochondria (see Eddy, 1988). Taking into account these structures, the mammalian sperm is morphologically structured in four structures; the head, the neck, the midpiece and the tail (see Figure 2).

The head is the apical section of the sperm cell. It contains four structures, the acrosome, the post-acrosomal dense plate, the sub-acrosomal space and the nucleus (Figure 2). Thus, this structure contains all of the genetic information that the male contribute to the future embryo, as well as the machinery that allows sperm to penetrate into the oocyte.

The acrosome is a vesicle located in the apical extreme of the head, covering the nucleus. This structure contains an amorphous material that corresponds with an enzymatic cocktail consisting in lythic enzymes designed to disaggregate the external oocyte structures during the sperm penetration. The acrosome is confined with a double cell membrane that is independent from the main cellular membrane, having a specific lipo-proteic structure derived from the Golgi apparatus (Eddy, 1988).

The post-acrosomal dense plate is an homogeneous plate composed with a electro-dense and fibrous material in which the inner acrosomal membrane is tightly fixed. Its location is at the distal apex of the acrosome and its function is the fixation of the acrosome until the acrosome reaction.

The sub-acrosomal space is the free space between the acrosome and the nucleus. It is especially wide under the apical zone of the acrosome, whereas it is practically absent in the lateral areas of the head between the acrosome and the nucleus. It is composed by cytoplasm and acts as a protective area for sudden changes of the external conditions.

Finally, the nucleus is the greatest and most important structure of the head. It is composed by DNA linked to specific nucleoproteins. The most important of these proteins is protamine, which can be present in two forms, the protamine 1 and the protamine 2 (Balhorn, 2007). The DNA/protamine structure is very tight, due to the fact that protamines are linked in the inner groove of the DNA helix (Biegeleisen, 2006). This originates a hypercondensed structure, which impedes any possibility of DNA expression. However, although protamines are the main proteins in the nucleus, there is also a significant proportion of histones (as much as the 15% of the total nucleoprotein content, see Wykes and Krawetz, 2003; O'Brien et al., 2005), indicating thus that a small proportion of the sperm nuclear structure would be similar to that of the somatic, eukaryotic cells. On the other hand, this strongly suggests that the sperm nucleus structure is heterogeneous, with main protamine-rich domains and concrete histone-rich domains, with an unknown function at this moment (Flores et al., 2011).

The sperm head connects in its distal apex with the neck or connecting piece. This small structure is, however, very important, since it has two key roles. The first role is to transmit the kinetic energy produced in the tail to the head, causing thus a progressive movement of the sperm. The second role is to transmit to the oocyte the centriolium that will be required to make the first cellular division after the fussion between both germ cells (Eddy, 1988). The neck contains then two types of structures. The first structures are all linked to the role of transmitting movement of the head. These structures, namely the basal plate, the laminar bodies, the capitulum and the segmented columns, are composed by fibrous, electrodense

material, and they are organized in a complex and rigid manner, enabling thus the movement transmission (Figure 3). The second main structure is the centriolium, as described above. It is similar to that any other centriolium observed in any somatic cell. Finally, the apical apex of the axoneme that will form the entire sperm tail is embedded into the distal zone of the neck (Figure 3), facilitating thus the transmission of the movement originated in the tail to the other sperm areas.

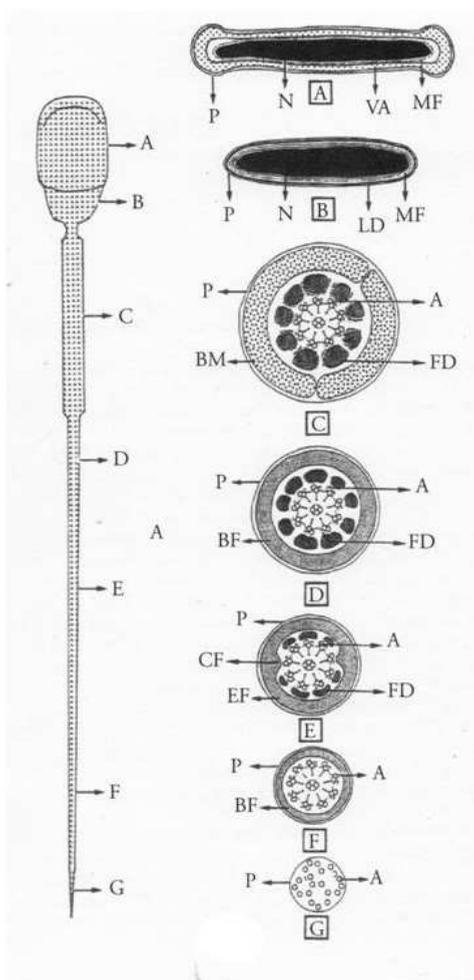


Fig. 3. Schematic representation of several transversal sections of a mammalian sperm indicating the present structures. A: Head, acrosomal area. B: Head, post-acrosomal area. C: Midpiece. D: Tail, proximal area. E: Tail, medial area. F: Tail, distal area. G: Tail, terminal area. MF: Perinuclear fibrous material. N: Nucleus. P: Cytoplasm. VA: Acrosome. LD: Postacrosomal dense plate. A: Axoneme. BM: Mitochondrial sheath. FD: Dense fibers. CF: Columns of the fibrous sheath. EF: Ribs of the fibrous sheath. Taken with permission from Bonet et al. (2000).

The structure that is placed immediately after the neck is the midpiece. This structure, in fact, corresponds to the apical zone of the tail, but it is characterized by the presence of a mitochondrial sheath that covers the inner tail structure (Eddy, 1988 and Figure 3). The mitochondrial sheath is composed by 150-200 mitochondria disposed in a helicoidal belt. These mitochondria are in a tight contact among them, although they are not fused together. This mitochondrial sheath finishes in its distal apex in a dense, annular structure known as the Hensen's ring (Figure 3). At this point, the midpiece finishes, giving place to the tail. It is not well known the exact role that this mitochondrial sheath plays in mammalian sperm function. A briefly discussion on this point will be made below. As an advance, we can say that several authors have suggested that the main role is the obtainment of energy for maintaining the sperm movement. However, recent data are not in agreement with this hypothesis. It is probable that other roles, such as the maintenance of a correct redox environment or the regulation of the capacitation could be more important than the suggested fuelling role.

The mitochondrial sheath of the midpiece involves the complete structure that will be continued in the tail. This structure is formed by a central axoneme with a structure similar to any other eukaryotic axonemes and a series of fibrous components that completely covers the axoneme (Eddy, 1988 and Figure 3). Of course, the axoneme is the main responsible for generating the sperm movement. However, the components that cover the axoneme are also very important, since they are responsible for transforming the axoneme movement in optimal for the sperm progressivity. The structures that cover the axoneme are basically a fibrous sheath and a variable number, in dependence of species, of longitudinal columns, which are a continuation of the fibrous columns that are present in the midpiece (Eddy, 1988 and Figure 3). As indicate above, these structures confers to the sperm movement its progressivity and planarity into the space. As a result, any defect in these structures will have disastrous consequences for the sperm movement. The final section of the tail, named terminal piece, is characterized by the lack of these complementary structures, leaving thus only the axoneme as the final sperm structure (Eddy, 1988 and Figure 3).

### **3.2 Functional characteristics of mammalian sperm**

Mammalian sperm have a myriad of specific functional characteristics, which are derived from their enormous specialization. A thorough study of these functional characteristics will need then the writing of a complete book, and here is not the place to make this. In this way, we will only made a brief introduction into one of the most intriguing questions regarding sperm function, the control of the energy metabolism and its relationship with the maintenance of motility.

Obviously, the control mechanisms of sperm energy management play an essential role. This is because of the fact that practically all of the reactions that maintain the functional status of the cell (control of tyrosine phosphorylation levels, maintenance of the membrane proteins glycosylation, etc.) need significant energy consumption. Thus, the optimal function of all of these mechanisms will depend, to a great extent, on a correct functioning of control mechanisms modulating sperm energy management. Unfortunately, and despite the great amount of knowledge that many investigators have accumulated in the past 15 years, there are several commonplaces regarding sperm energy metabolism, which, in fact, obstruct an optimal, practical application of this knowledge. Thus, everybody knows that

the spermatozoon is a totally strict, glycolytic cell. Of course, this is an unquestionable fact. However, the adoption of this assertion, without any doubt, can lead to the opinion that spermatozoa are almost exclusively glycolytic so, then, they have practically no other modulator system to manage their energy levels (Mann 1975). On the other hand, if the spermatozoon is an exclusively glycolytic cell, what is the role of sperm mitochondria and the associated Krebs cycle? In this respect, it is noteworthy that many investigators indicate as an absolute fact that the energy obtained through the Krebs cycle is, in all conditions, absolutely necessary for the maintenance of sperm motility in all species (Nevo et al. 1970; Ford and Harrison 1985; Halangk et al. 1985; Folgero et al. 1993; Ruíz-Pesini et al. 1998), despite the same investigators maintaining an absolute pre-eminence of glycolysis to obtain sperm energy, without noticing the energy contradiction that the simultaneous assumption of both principles implies. These contradictions highlight the complexity of the question, which has to be approached with an open mind. Only in this manner some valid and general conclusions with practical applicability on sperm conservation should be attained.

Once monosaccharides have been uptaken, transformed and phosphorylated to obtain glucose 6-phosphate (G 6-P), they undertake the appropriate metabolic pathway designed for energy synthesis. If we preclude any possible anabolic pathway (glycogen synthesis, pentose phosphate cycle, etc.; see Urner and Sakkas 1999; Ballester et al. 2000), the utilization of G 6-P to obtain ATPs starts with the glycolytic pathway, which culminates in the obtainment of pyruvate. Afterwards, the obtained pyruvate can be sent either to the formation of extracellular lactate or to its introduction into the mitochondrial Krebs cycle. This last step is controlled by the lactate dehydrogenase (LDH) activity, and it is noteworthy that the further metabolization of pyruvate/lactate through the Krebs cycle yields a great amount of ATPs, as well as important levels of reducing potential in the form of nicotinamide adenine dinucleotide hydrogenase (NADH). This NADH is of great importance not only in the maintenance of anabolic pathways, but also in the control of intracellular redox and pH levels. The equilibrium between sugar metabolization through simple glycolysis or through glycolysis plus the Krebs cycle depends on a great number of factors, such as the O<sub>2</sub> pressure, the pH, the intracellular levels of ATP and the action of several intracellular signalling factors, like nitric oxide (Stryer 1995). All of these factors allow for a very fine regulation in order to maintain the appropriate ATP intracellular levels and, thus, the required sperm energy levels at each point of its life-time. If we analyse the published data regarding the glycolysis/Krebs cycle equilibrium in mammalian sperm, we can observe the existence of clearly contradictory results, as indicated above. Thus, there is a general consensus about the fact that practically all mammalian sperm from fresh ejaculates has very high glycolytic activity (it can comprise more than 95% of the formed ATP in boar sperm from fresh ejaculates; see Marín et al. 2003). This very high glycolytic rhythm is one of the main factors that preclude the obtainment of a stable, estequiometric equilibrium in this pathway (Hammersted and Lardy 1983). Nonetheless, several authors have described that the energy obtained from the Krebs cycle is absolutely necessary to maintain sperm motility in species such as bull, despite the fact that the energy obtained from this pathway is very small (Nevo et al. 1970; Ford and Harrison 1985; Halangk et al. 1985; Folgero et al. 1993; Ruíz-Pesini et al. 1998). To complicate this question further, other authors have described that, in fact, the energy from the Krebs cycle is not necessary to maintain motility in other species like mice (Mukai and Okuno 2004). How can all of these contradictory results have a global meaning?

As in other points, we can only speculate about the exact meaning of the data shown above. In spite of this, some points can be discussed in order to clarify this fundamental aspect of sperm energy management. As a first question, we must consider the hypothesis of the existence of separate metabolic phenotypes in mammalian sperm (Rodríguez-Gil, 2006). If we consider this hypothesis, we can also suggest that each metabolic phenotype will have a separate equilibrium between the catabolic and the anabolic pathways. These different equilibriums will lead to concomitant differences in the preponderance of the principal energy obtaining pathways, whether anaerobic (glycolysis) or aerobic (Krebs cycle). In this sense and concerning fresh ejaculates, dog sperm, which has a very active glycogen metabolism concomitantly to high intracellular G 6-P and other hexose 6-phosphate levels, shows a mitochondrial oxidative activity higher than boar spermatozoa, which have a much less active glycogen metabolism and practically absent G 6-P levels (Rigau et al. 2002; Marín et al. 2003; Medrano et al. 2006a). This difference implies that the equilibrium between glycolysis- and Krebs cycle-obtained ATPs will be very separate between both species and, as a consequence, the specific regulation of both pathways would also differ. Following this comparison, the existence of species in which ATPs from Krebs cycle would be absolutely necessary to maintain motility is easily assumable whereas ATPs of a similar origin would be of a much lesser importance in other mammals. Another possible explanation for the observed discrepancies can be related to the fact that authors have ignored that mammalian sperm is a cell with a very active and complex life-cycle, with enormous changes in its specific function during its life-time. Thus, we can only bear in mind that sperm motility in sperm from fresh ejaculates in all species is very different to that from the hyperactivated sperm subjected to capacitation (Yanagimachi 1994b). Therefore, if we assume that capacitated sperm has energy requirements far greater than those from recently ejaculated cells in order to maintain hyperactivated motility and all of the energy consuming processes linked to acrosome reaction and oocyte penetration, it is logical to assume that the energy-obtainment mechanisms will change during capacitation to obtain a higher energy-production rhythm. This implies that a direct comparison of the energy obtained from glycolysis alone and the Krebs cycle in freshly obtained and capacitated sperm from the same ejaculate will show very great differences between each other. A clear example of this point is boar spermatozoa, where the proportion of energy that is obtained from the Krebs cycle in cells from fresh ejaculates is very low, less than 5% of the total generated energy (Marín et al. 2003). However, other experiments show that the attainment of "in vitro" capacitation induces a constant increase in the mitochondrial activity of these cells, which was measured through specific staining and analysis of O<sub>2</sub> consumption (Ramíó-Lluch et al., 2011). These data indicate that mammalian sperm has the ability to equilibrate its energy-obtainment systems depending on its necessities. These necessities will surely be modulated by factors such as variations in the intracellular levels of ATP and other related nucleotides (ADP and AMP), since these levels tend to be maintained within very narrow limits, at least those of ATP, in separate species subjected to different study conditions (Rigau et al. 2002; Medrano et al. 2006a). This ability causes the direct comparison of the results obtained from separate laboratories to be very difficult, since the small differences that each laboratory will introduce into its precise methodology can result in great effects on the specific energy equilibrium. This can also to be a partial explanation of the contradictions shown in the bibliography that have greatly hampered a global comprehension of the mammalian sperm energy-levels management.

## **4. Technical approaches to the global study of whole cell function: metabolomics and proteomic arrays**

### **4.1 Metabolomics**

In the last years, a series of novel techniques have been developed in order to obtain a more global vision of the overall cell function. In this sense, specialties such as metabolomics have been applied with success in many cellular systems. Metabolomics has been defined as the study of the chemical processes in a cell involving the processing of metabolites as a whole. This discipline allows investigators a more comprehensive knowledge of all the processes related to the obtainment of energy in a whole cell. This is especially important in mammalian sperm, in which the metabolization of energy substrates is not only related to the obtainment of the energy, but also with the regulation of other cellular processes, like phospho-dephosphorylation of proteins not directly related with the energy regulation (Urner & Sakkas, 2003). Thus, metabolomics is a very important tool in the interpretation of the regulation of mammalian sperm function.

However, the application of metabolomics in mammalian sperm requires several particularities, linked not only to the specific characteristics of sperm energy metabolism, but also to the specific morphological characteristics of sperm cells. It must be reminded that mammalian sperm present morphological characteristics that difficult in a great measure their processing for the analysis of any molecular mechanisms. One of the most important of these characteristics, as it has been indicated above in this chapter, is the very great percentage of non-soluble structures integrating the whole cell. Some of these structures are the nucleus, the axoneme and even the mitochondrial sheath, which are mostly composed by very tightly components (hyper stabilized DNA, axoneme proteins, fibrous sheath and longitudinal columns proteins, etc, see Klaus & Hunnicutt, 2006) These proteins are very difficult to isolate and even separate from other components of the sperm structure. This greatly difficult the correct processing of all of the molecules related with these sperm structures which, in turn, impedes in many occasions to reach to correct interpretations of the data yielded after this processing. Furthermore, the correct processing of semen samples would be different depending on the structure or the specific functional aspect that was studied. As an example, the study of membrane-linked functions such as uptake of metabolites will require a sample treatment in which membrane components will be detached from the rest of sperm structures, usually combining the homogenization of samples with the addition of a detergent in the homogenization buffer, and, in some cases, the study will further require a subsequent enrichment of the homogenized samples through ultracentrifugation. On the contrary, the study of some of sperm nuclear function will require a much harder sample treatment, in order to release nuclear components that are very tightly bounded among them, such as protamines. Other important question that has limited the use of metabolomic approximations to the study of the sperm function is the high economical cost needed for these studies. This cost is basically due to that metabolomics requires the utilization of sophisticated equipment, such as mass-spectrophotometric analyzers and the work of expert personnel in the computerized analysis of data obtained from the rhythm of variations of isotopic levels in treated samples. This implies that these works are not accessible to investigators and in the majority of times the co-operation among several interdisciplinary groups is mandatory. As a consequence, there are very few manuscript published until now in which metabolomics has been applied

to the study of sperm functionality, and the following explanation will be based in the work published in Marín et al. (2003).

The most important question arising from a metabolomic study is to determine the fate of an specific substrate when it is processed by cell and the evaluation of putative changes in the rhythm of pathway in which this substrate is processed when cell function changes. Centering on the sperm studies, they have been centered in determining the fate of both glucose and lactate as an energy source of freshly obtained boar sperm (Marín et al., 2003). Taking into account this purpose, metabolomic studies was carried out through the combination of three separate techniques. The first technique was a conventional analysis of intracellular levels of the perhaps most important intermediate glucose metabolite, G 6-P. The second technique was the determination of the rhythm of glucose oxidation, a marker of the Krebs cycle, through incubation of sperm with the randomly  $^{14}\text{C}$  radioactive substrates [ $\text{U-}^{14}\text{C}$ ] glucose and [ $\text{U-}^{14}\text{C}$ ] lactate and the subsequent analysis of the  $^{14}\text{CO}_2$  formation. The third technique was the mass isotopomer analysis by gas chromatography/mass spectrometry (GC/MS) of the intermediate metabolites originated after the incubation of boar sperm with the no-radioactive substrate [ $1,2\text{-}^{13}\text{C}_2$ ]glucose (Marín et al, 2003).

#### **4.1.1 Determination of glucose 6-phosphate intracellular levels**

As commented above, the determination of the intracellular levels of G 6-P is carried out through a spectrophotometric technique, described in Michal (1984). Briefly, it consists in two successive steps. The first step is the homogenization of samples. The second step is the incubation of samples with the appropriate substrates to obtain an optically active product, and its determination by spectrophotometry.

Regarding the homogenization of samples (Fillat et al., 1992; Marín et al., 2003), this procedure must separate low-molecular weight, soluble metabolites such as sugars from all of the other cell components with a greater molecular weight, like nucleic acids or proteins. For this purpose, samples will be homogenized in the presence of acid, in order to precipitate all of the high-molecular weight components. The most common acid utilized for this purpose is an aqueous solution of perchloric acid ( $\text{HClO}_4$ ) at a concentration of 10% (v:v). Homogenization can be carried out by using different systems, like sonication or mechanical rupture, either through a manual technique or an automatized one. However, in this precise technique, the homogenization system is not as crucial as that in other techniques devoted to the obtainment of other cell components, such as proteins, since the existence of a very acidic environment will cause the precipitation of the majority of the cell components by itself. One vital point to consider, however, is that the homogenization has to be carried out at  $4^\circ\text{C}$ , since greater temperatures can cause a rapid destruction of the soluble metabolites that we want to determine. Thus, the  $\text{HClO}_4$  solution has to be stored and utilized at  $4^\circ\text{C}$ , and the homogenization technique has to be performed at this temperature (Rigau et al., 2002; Medrano et al., 2006a). The  $\text{HClO}_4$  volume that is added to the samples varies depending on the cell concentration, although it is usually in a range between 200  $\mu\text{L}$  and 500 $\mu\text{L}$ . After homogenization, samples will be subjected to centrifugation at 29,000 g for 5 minutes at  $4^\circ\text{C}$ , and clear supernatants will be collected, carefully avoiding a possible mechanical remixing with the obtained pellet (Fillat et al., 1992; Marín et al., 2003).

At this moment, low-molecular weight metabolites like G 6-P will be contained in the clear supernatant. Notwithstanding, they are solubilized in a very acidic medium, in which their structural stability is very short. To avoid this, samples have to be immediately neutralized after their obtainment. For this purpose, the obtained supernatants will be added with a low volume (i.e., 5  $\mu\text{L}$ ) of any sort of liquid pH indicator, which suffers a color change depending on the medium pH. Immediately afterwards, we will add to the samples very low volumes of a concentrated solution of a strong basic solution. The most common solution is an aqueous one of 5M  $\text{K}_2\text{CO}_3$ , which will yield a no-soluble  $\text{KClO}_4$  precipitate when it reacts with the excess of the  $\text{HClO}_4$  in the medium (Fillat et al., 1992; Marín et al., 2003). The addition of the  $\text{K}_2\text{CO}_3$  must be performed very slowly and in very small quantities (i.e., volumes of about 5  $\mu\text{L}$ ), and, after the addition of one of these small volumes, samples will be mixed and the color of samples will be observed. When samples color will indicate that they have reached a pH of about 7, we will stop the addition of  $\text{K}_2\text{CO}_3$  and the exact volume of the added basic solution will be annotated in order to determine the exact, final volume of the sample (Fillat et al., 1992; Marín et al., 2003). Finally, the neutralized will be subjected to another centrifugation at 29,000 g for 15 minutes at 4°C in order to eliminate the produced  $\text{KClO}_4$  pellets, and supernatants will be immediately subjected to the G 6-P determination. It is noteworthy that the stability of the obtained, neutralized samples is not very great. In fact, samples can be utilized only for a few hours after their obtainment, and they must be placed in this time at 4°C. These samples can not be conserved frozen for a long time also, and they should be processed as longer as 2-3 days after their obtainment.

The most usually spectrophotometric technique utilized for determining G 6-P levels is that based in the ability of G 6-P to be oxidized by the enzyme glucose 6-phosphate dehydrogenase (G 6-P DH, see Michal, 1984). This reaction will be yield in the presence of  $\text{NAD}^+$ , which will be reduced to NADH. The NADH is an optically active substance at a wavelength of 340 nm, and, in this way, the absorbance change at 340 nm will be a direct result of the presence of G 6-P in the sample. The technique, based in that published in Michal (1984), is performed as follows:

An aliquot of 300  $\mu\text{L}$  will be mixed with 225  $\mu\text{L}$  of a reaction mixture of an adjusted buffer (Ph 7.4) containing 0.1M Tris, 1M  $\text{Cl}_2\text{Mg}$  and 20  $\mu\text{g}/\mu\text{L}$   $\text{NAD}^+$ . The mixture will be incubated for 2 minutes at 37°C. Afterwards, a first optical lecture will be made to obtain the initial absorbance of the sample ( $\text{\AA}_0$ ). After this lecture, the mixture will be added with 15  $\mu\text{L}$  of a solution of G 6-P DH with a specific activity of 30 U/mL. This will be mixed and further incubated for 10 minutes at 37°C. After this time, a final optical lecture will be made to obtain the final absorbance of the sample ( $\text{\AA}_1$ ). The G 6-P levels of the sample will be obtained then through the difference between  $\text{\AA}_1$  and  $\text{\AA}_0$ , after applying the logical corrections with the appropriate G 6-P standards and the correction for the intrinsic absorbance of the sample. Finally, G 6-P levels will be normalized through the determination of the total protein content of samples. This will be determined in the pellets obtained after the homogenization in the presence of  $\text{HClO}_4$ . These pellets will be resuspended in 400  $\mu\text{L}$  of 1M  $\text{K}_2\text{CO}_3$  and heated at 60°C until the resuspension of pellets. The total protein content of these resuspended pellets will be determined through the Bradford method (Bradford, 1976), by using a commercial kit, in our case from BioRad (Hercules, CA).

#### 4.1.2 Determination of the rhythm of formation of $^{14}\text{CO}_2$ formation from [U- $^{14}\text{C}$ ]-marked substrates

In this technique, spermatozoa until a final volume of 250  $\mu\text{L}$  will be incubated in a standard Krebs-Ringer-Henseleit medium added with the [U- $^{14}\text{C}$ ] marked substrate (Rodríguez-Gil et al., 1991; Marín et al., 2003). In the sperm metabolomics works published until now, the utilized substrate is [U- $^{14}\text{C}$ ]-glucose at a final concentration of 10 mM (Marín et al., 2003). In all cases the specific radioactivity will be of 5000 cpm/ $\mu\text{mol}$  substrate. The incubation will be carried out in Eppendorf tubes in which a small piece of a standard filter paper totally soaked in  $\beta$ -phenylethylamine will be cased into the tap of the tube. The  $\beta$ -phenylethylamine is a compound that will trap all of the  $\text{CO}_2$  that will be released by the cells. It is essential to avoid that the sperm cell suspension will contact this piece of paper. Eppendorf tubes will be closed as tightly as possible and then cells will be subjected to incubation during 60 minutes at  $37^\circ\text{C}$  in very gentle shaking. Again, this shaking will be enough gentle to avoid the contact between filter papers and cell suspensions. After this time, cells will be killed by the addition of 350  $\mu\text{L}$  of 10 % (v:v)  $\text{HClO}_4$ . The acid will also release all of the  $\text{CO}_2$  that will be accumulated into the cells, including the  $^{14}\text{CO}_2$  obtained after the cellular processing of the  $^{14}\text{C}$ -marked utilized substrates. Samples will be further incubated for 30 additional minutes at  $37^\circ\text{C}$  in gentle shaking, which will allow for a complete release of the intracellular  $\text{CO}_2$ . Afterwards, the  $\beta$ -phenylethylamine-soaked filter papers of the tubes will be gently extracted, avoiding again any contact with the liquid of the tubes. These papers will be left to air dry into a laminar flux chamber. Once dried, the radioactivity presented in the filter papers will be determined by using a liquid scintillation counter previous immersion of papers in a vial containing 2,5-diphenyl oxazol. Afterwards, the rhythm of substrate oxidation by cells will be easily calculated taking into account the proportion of radioactive counts found in the papers when comparing with the total radioactive counts placed in the incubation medium.

#### 4.1.3 Determination of the rhythm of formation of intermediate metabolites through gas chromatography/mass spectrometry

The utilization of [U- $^{14}\text{C}$ ]-marked substrates only allows investigators to determine the final fate of the substrates after their metabolization by the cells. However, this technique does not allow investigators to know what are the exact metabolism pathways by which sperm are able to metabolize these substrates. This will impede a global survey of the metabolic pathways utilized by spermatozoa to utilize these substrates through the analysis with [U- $^{14}\text{C}$ ] marked substrates. However, this problem can be overcome if investigators are able to utilize marked substrates but not in a uniform manner, but with substrates in which marking is linked to one specific carbon radical. Moreover, if the utilized marked substrate is not radioactive, all of the problems that are inherent to their utilization will be eliminated and, in this way, the handling of experiments will be much easier. This is possible if investigators can utilize  $^{13}\text{C}$ -marked substrates, which can be analyzed through GC/MS. Centering on the results published regarding sugar utilization pathways by boar spermatozoa (Marín et al., 2003), the chosen marked substrate was the no-radioactive [1,2- $^{13}\text{C}_2$ ]-glucose. This specific marking allows investigators to elucidate the proportion of substrate that is metabolized by boar sperm cell through their entry into the glycolytic pathway, the accumulation of this marked glucose into glycogen and even the pass of marked glucose through the other possible metabolic pathways, namely pentose phosphate

cycle and Krebs cycle after the glycolytic pathway. Likewise, this specifically marked substrate is able to determine the percentage of glucose that can be derived to the synthesis of fatty acids. Finally, a putative reconversion of glucose into gluconeogenesis, which will be linked with an indirect pathway for glycogen synthesis, can also be determined following the fate of the [1,2-<sup>13</sup>C]-glucose. The Figure 4 shows how the [1,2-<sup>13</sup>C<sub>2</sub>]-glucose can be tracked for all their putative metabolic pathways in the cell. This Figure is excerpted from Marín et al. (2003).

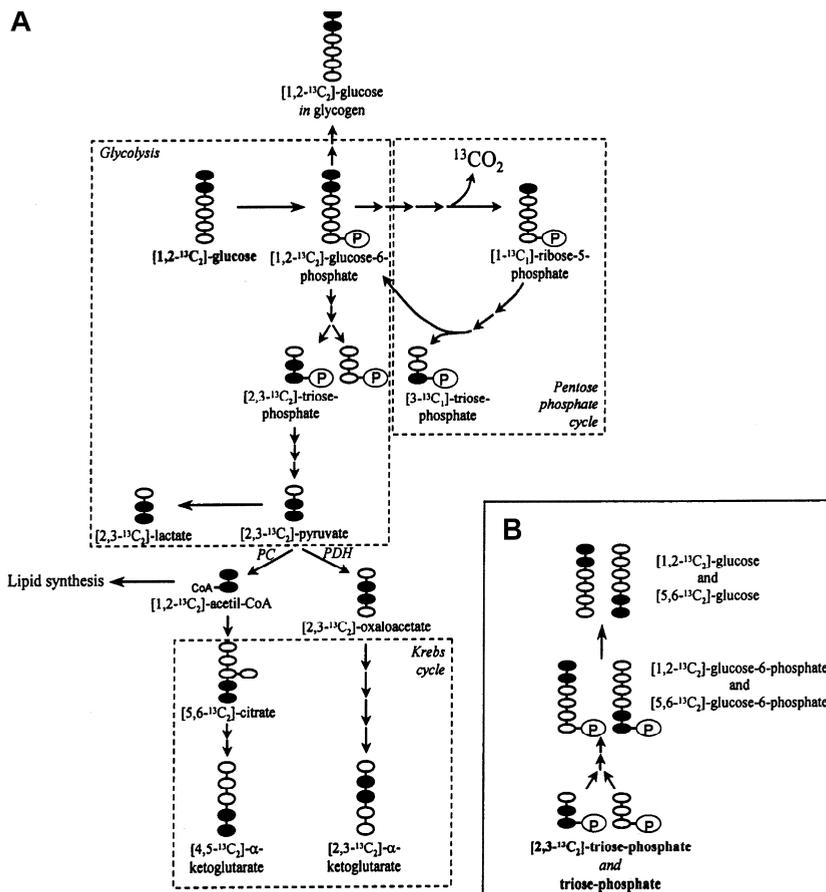


Fig. 4. Expected mass isotopomers in lactate, ribose, newly synthesized fatty acids, and glutamate from incubations with [1,2-<sup>13</sup>C<sub>2</sub>]-glucose (A) and in newly synthesized glucose from gluconeogenesis (B). When [1,2-<sup>13</sup>C<sub>2</sub>]-glucose enters into the cell, it is converted into glucose-6-phosphate (G6P), which can undergo glycogen synthesis, and enter the glycolytic pathway or the pentose phosphate cycle (PPC). From glycolysis, two triose-phosphate molecules are formed, one of them with two <sup>13</sup>C and the other one without <sup>13</sup>C. Both can then form pyruvate, and therefore 50% [2,3-<sup>13</sup>C<sub>2</sub>]-lactate. Pyruvate can also enter lipid synthesis, forming molecules with a paired number of <sup>13</sup>C atoms, or the Krebs cycle, obtaining two different labelling distributions in α-ketoglutarate (which is in equilibrium

with medium glutamate) depending on the enzyme used to enter the Krebs cycle: pyruvate carboxylase (PC) or pyruvate dehydrogenase (PDH). When G6P enters the PPC, one  $^{13}\text{C}$  is lost in  $\text{CO}_2$  formation, giving ribose-5-phosphate with only one labelled atom. This molecule can also enter the non-oxidative pentose phosphate pathway forming triose-phosphate molecules with only one  $^{13}\text{C}$ , and all subsequent products labelled in one atom. Furthermore, when products from glycolysis of  $[1,2-^{13}\text{C}_2]$ glucose undergo gluconeogenesis, two different labelling patterns are expected in glucose isotopomers:  $[1,2-^{13}\text{C}_2]$ glucose, which is the initial isotope, and  $[5,6-^{13}\text{C}_2]$ glucose, formed as a result of the isotopic equilibrium between the labelled and unlabeled triose-phosphates.

The application of the GC/MS requires a previous preparation of the samples that will be different, depending upon the isotopes that investigators are looking for. In the case of glucose metabolites, the preparation of samples will start with the extraction from semen samples of the low-molecular weight. This will be done through the homogenization of samples until obtaining a perchloric acid-pH adjusted supernatant likewise to that described for the determination of G 6-P levels (Fillat et al., 1992). Once obtained this extract, samples will be treated as described in Tserng et al. (1984); Lee et al. (1996) and Kurland et al. (2000). For this purpose samples will be taken to dryness and they will be subsequently resuspended in 400  $\mu\text{L}$  of 0.5 % (w:v) hydroxylamine hydrochloride in pyridine. The mixture will be heated to 100 °C for 1 h and will be then evaporated to dryness under nitrogen. To this residue will be added 100  $\mu\text{L}$  of pyridine and 20  $\mu\text{L}$  of acetic anhydride. The solvent and excess reagent will be removed by evaporation under nitrogen, and the obtained pellet will be dissolved in 20-50  $\mu\text{L}$  of pyridine for the GC/MS analysis. The GC/MS induces the chemical ionization of the components of the sample. This ionization activates molecules for the GC/MS detectors, and the activated- $^{13}\text{C}$ -marked compounds can be fractionated and further detected through the mass spectrophotometry system. As indicated in Kurland et al. (2000) for the determination of glucose derivatives, the GC conditions will be of a 6 feet X 2-mm, inner diameter glass column packed with 3% SP2340 on 100/120 Supelcoport. The helium flow rate will be of 20 mL/min at a column temperature of 235°C. The retention time for glucose derivative will be of 2.4 min, and chemical ionization will be performed by using methane as the carrier gas. This treatment will create active, detectable forms in the range of 327-336 m/z. This range will include  $^{13}\text{C}$ -marked glucose and all of their derivatives (Marín et al., 2003). The molar ratios of the isotopomers included in this range will be calculated from the ion intensities using a weighted multiple linear regression analysis (Hammersted & Lardy, 1983). The distribution of isotopomeric species will be expressed as molar fractions of the total glucose concentration. The percent content of  $[^{13}\text{C}]$ glucose will be calculated as the weighted average  $^{13}\text{C}$  content of the isotopomers.

## 4.2 Proteomic arrays

Whereas metabolomics allows investigators to elucidate the pathway/s by which a substance is utilized by a cell, arrays allows investigators to determine the global status of a specific cell function through estimation of either the specific amount of the proteins involved in this function or the phosphorylation/glycosylation levels of these proteins. In this way, investigators can have a global survey of the exact situation of a specific molecular mechanism in the moment of the analysis. There are, of course, other types of arrays than

those devoted to the study of proteins. In this sense, microarrays are widely utilized for the study of global genic expression and location, and they are the basis of the named genomics. However, genomics is not a very useful tool in the study of mature sperm function, since these cells do not have gene expression (Ward & Coffey, 1991), and their response to both external and internal stimuli are totally based in changes in the degradation/loss, location and post-translational structure, without any possibility to arrange newly synthesized proteins from their nuclear machinery. Thus, the study of a local sperm cell function mechanism has to be approached by using protein-devoted miniarrays, following the discipline named as proteomics.

There are in the literature many examples of proteomic studies in many cells and tissues. However, sperm proteomics is practically absent. At this moment, we can only detected in the literature one article, regarding changes in serine, tyrosine and threonine phosphorylation levels of a wide arrays of proteins involved in the control of the global sperm function, like protein kinases and phosphatases in both dog and boar mature sperm subjected to incubation with either glucose or fructose (Fernández-Novell et al., 2011). This article, however, can be a good basis for describing the application of the proteomic arrays in sperm as follows.

First of all, it is noteworthy that proteomic arrays are commercial products manufactured and commercialized by several different commercial firms. The precise technique to manufacture these arrays is, of course, subjected to patent protection and, in this manner, this is not possible to offer a detailed explanation of the technique utilized for arrays manufacture. Taking into account this limitation, a miniarray consists in an inert basis. This basis is usually a small square of a material similar to that utilized in the protein transferences in the Western blotting techniques, although other materials can be also utilized. Manufacturers placed in very concrete points of these arrays a known amount of a specific antibody against one protein of interest, in a manner in which the putative antigen-antibody reaction that investigators will be look for will located in a small point onto the inert basis. Thus, the basis can be full by a great number of small points in which specific antibodies for one protein can be placed (see Figure 5).

Once the studied proteins have been immobilized onto the array through their linking to their specific antibody, investigators can study these proteins through two different ways. The first way is the analysis of the amount of each protein. This is done through a direct developing of the performed antigen-antibody reaction through a system equal to that carried out in the Western blotting analysis with the transferred samples. In fact, as indicated above, the miniarray is similar to a transferred membrane of a Western blotting analysis and, thus, it can be analyzed in the same manner. The second way is the analysis of post-translational modifications of the proteins immobilized on the arrays after the antigen-antibody reaction. For this purpose, the miniarrays on which the selected proteins have been fixed are subsequent incubated with a specific protein from a concrete post-translational modification mechanism. The most common post-translational mechanisms that control protein function are the changes in the phosphorylation levels of these proteins (Isen et al., 2006). Protein phosphorylation can be only be made on serine, tyrosine or threonine residues (Isen et al., 2006), and specific antibodies against protein serine, tyrosine and threonine phosphorylation have been developed by several commercial firms. Thus, membranes are incubated in the presence of one of these specific antibodies in order to

analyze the amount of serine, tyrosine or threonine phosphorylation of all of the proteins previously immobilized there. Afterwards, results are developed in the same way that as for the Western blotting technique (Burnette, 1981), since, in fact, both systems are very similar.

Taking into account all of these information, the miniarrays techniques that has been published regarding mature sperm function have been carried out under the following procedure (Fernández-Novell et al., 2011).

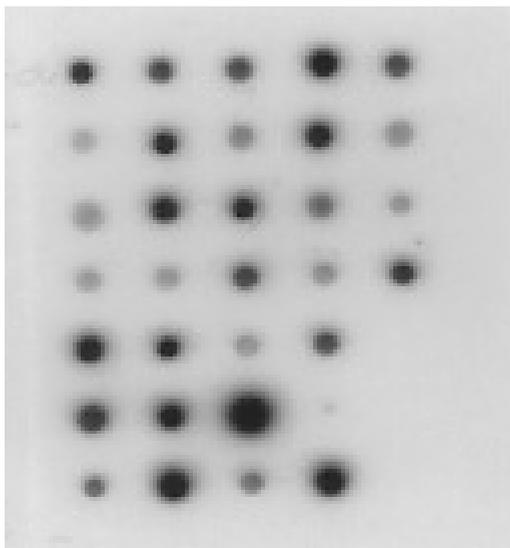


Fig. 5. Aspect of a proteomic miniarray in which positive spots indicate the presence of a specific antigen-antibody reaction between the specific antibody placed onto the inert base and the antigen present in the sample placed on the array. This image has been taken from Fernández-Novell et al. (2011).

Sperm samples are homogenised in 1mL of an ice-cold extraction solution comprising a 15mM Tris/HCl buffer (pH 7.5) plus 120mM NaCl, 25mM KCl, 2mM EGTA, 2mM EDTA, 0.1mM DTT, 0.5% Triton X-100, 10 mg/mL leupeptin, 0.5mM PMSF and 1mM Na<sub>2</sub>VO<sub>4</sub> (extraction solution). Of these buffer components, EGTA and EDTA are ion chelants that can act as protein phosphatases inhibitors. Another protein phosphatase inhibitor is Na<sub>2</sub>VO<sub>4</sub>, thus avoiding artifactual changes in the phosphorylation levels of the studied proteins. The detergent Triton X-100 facilitates sperm homogenization through cell membrane lysis, whereas leupeptin and PMSF are known protease inhibitors (Roche Applied Science, 2004), avoiding thus the artifactual decrease of the sample protein content. Afterwards, homogenised samples are left for 30 min at 4°C and then centrifuged at 10,000g for 15 min at 4°C. Supernatants are taken and then used to test the degree of tyrosine, serine and threonine phosphorylation of selected proteins included in the chosen arrays, which is purchased to a commercial firm that followed a custom array of tested proteins. The analysis is performed following the standard protocol provided by the manufacturer. Briefly, each sample is diluted in 2mL of an extraction solution supplied by the commercial firm and containing 1% dry milk to reach a final protein concentration of 2 mg/mL.

Simultaneously, the supplied array membranes containing the specific antibodies are placed in standard 60mmx15mm suspension culture dishes and incubated with a blocking solution containing 150mM NaCl, 25mM Tris and 0.05% (v/v) Tween-20 (TBST; pH 7.5) plus 5%(w/v) dry milk and left for 1 h at room temperature under slow shaking. Membranes are then incubated with the samples for 2 h at room temperature under slow shaking. After incubation, membranes are washed 3 times, 15 minutes each with TBST. Afterwards, the membranes are incubated with 10 mg/mL of an HRP-conjugated antibody against serine, tyrosine or threonine phosphorylated proteins diluted in TBST for 2 h at room temperature under slow shaking. Finally, samples are washed 3 times, 15 minutes each with TBST and then incubated with a peroxidase substrate and exposed to a commercial X-ray film. Concomitantly, the total protein content of the supernatants is determined by the Bradford method (Bradford, 1976) using a commercial kit from BioRad. The intensity of the spots obtained is quantified using any specific software for image analysis of blots and arrays, in which background has to be previously made uniform for all of the arrays analysed. The values obtained for the control samples (i.e. those incubated in the absence of sugars) have to be transformed in order to obtain a basal arbitrary value of 100, from which the intensity values for the other samples will be calculated. Furthermore, two types of negative control must be applied. In one, one or two arrays must be incubated with a randomly chosen sample but without further incubation with the primary antibody. In the other negative control, one or two arrays have to be incubated with the antibodies but without samples. Statistical differences between groups can be checked by using the Student-Neumann-Keuls test or any of their derivatives. However, since the analytical technique has an intrinsic subjective component, investigators must consider that true differences would be only considered as those for which a  $P0.05$  and a percentage difference above 20% were detected.

## 5. Conclusion

The study of mammalian sperm biology is a very open field with many unanswered questions at this moment. This is mainly due to the fact that mammalian sperm have a very complex life, which is translated in the existence of many, concomitant and complex molecular mechanisms that control the whole sperm function. The overall study of this complexity is not completely possible with the classical analytical tools, based on the analysis of concrete, punctual functional aspects. This is also true for immunocytochemistry, which has been mainly devoted to the location of specific proteins in the study of sperm function. In this way, only the application of integrated analytical systems, such as the metabolomics or the miniarray studies can achieve a better and deeper knowledge of the mature mammalian sperm functionality. In this book, we proposed that the coordinated utilization of these integrated analytical systems was named "spermiomics". In this manner, spermiomics would be the best tool for future investigations of the overall, mature mammalian sperm function.

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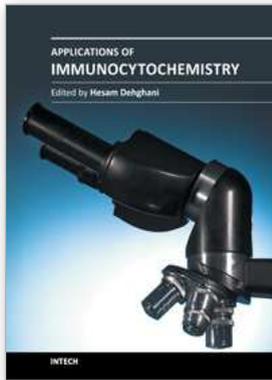
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## **Applications of Immunocytochemistry**

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Immunocytochemistry is classically defined as a procedure to detect antigens in cellular contexts using antibodies. However, over the years many aspects of this procedure have evolved within a plethora of experimental setups. There are different ways to prepare a given specimen, different kinds of antibodies to apply, different techniques for imaging, and different methods of analyzing the data. In this book, various ways of performing each individual step of immunocytochemistry in different cellular contexts are exemplified and discussed. Applications of Immunocytochemistry offers technical and background information on different steps of immunocytochemistry and presents the application of this technique and its adaptations in cell lines, neural tissue, pancreatic tissue, sputum cells, sperm cells, preimplantation embryo, arabidopsis, fish gonads, and Leishmania.

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