Alma Mater Studiorum – Università di Bologna

Dottorato Di Ricerca in

Morfofisiologia veterinaria e applicazioni biotecnologiche

Ciclo XXII

SSD: VET 02

Detection and localization of GLUTs in spermatozoa from different domestic species

Presentata da
Diego Bucci

Coordinatore Dottorato
Prof. Eraldo Seren

Docente Guida
Prof. Carlo Tamanini

Correlatore
Dr.ssa Marcella Spinaci

Esame finale anno 2010

Abstract

Sperm cells need hexoses as a substrate for their function, for both the maintenance of membrane homeostasis and the movement of the tail. These cells have a peculiar metabolism that has not yet been fully understood, but it is clear that they obtain energy from hexoses through glycolisis and/or oxidative phosphorylation.

Spermatozoa are in contact with different external environments, beginning from the testicular and epididymal fluid, passing to the seminal plasma and finally to the female genital tract fluids; in addition, with the spread of reproductive biotechnologies, sperm cells are diluted and stored in various media, containing different energetic substrates. To utilize these energetic sources, sperm cells, as other eukaryotic cells, have a well-constructed protein system, that is mainly represented by the GLUT family proteins. These transporters have a membrane-spanning α-helix structure and work as an enzymatic pump that permit a fast gradient dependent passage of sugar molecules through the lipidic bilayer of sperm membrane.

Many GLUTs have been studied in man, bull and rat spermatozoa; the presence of some GLUTs has been also demonstrated in boar and dog spermatozoa.

The aims of the present study were

- to determine the presence of GLUTs 1, 2, 3, 4 and 5 in boar, horse, dog and donkey spermatozoa and to describe their localization;
- to study eventual changes in GLUTs location after capacitation and acrosome reaction in boar, stallion and dog spermatozoa;
- to determine possible changes in GLUTs localization after capacitation induced by insulin and IGF stimulation in boar spermatozoa;
- to evaluate changes in GLUTs localization after flow-cytometric sex sorting in boar sperm cells.

GLUTs 1, 2, 3 and 5 presence and localization have been demonstrated in boar, stallion, dog and donkey spermatozoa by western blotting and immunofluorescence analysis; a relocation in GLUTs after capacitation has been observed only in dog sperm cells, while no changes have been observed in the other species examined. As for boar, the stimulation of the capacitation with insulin and IGF didn’t cause any change in GLUTs localization, as well as for the flow cytometric sorting procedure.

In conclusion, this study confirms the presence of GLUTs 1, 2 ,3 and 5 in boar, dog, stallion and donkey spermatozoa, while GLUT 4 seems to be absent, as a confirmation of other studies. Only in dog sperm cells capacitating conditions induce a change in GLUTs distribution, even if the physiological role of these changes should be deepened.

Introduction

Studying sperm: when it began

Sperm cell study, nowadays better known as spermatology, finds its roots in the first observations, carried out by Antonius Leeuwenhoek, in 1677. This scientist, with the help of the microscope, discovered “little animals” swimming in seminal fluid. The fascination of the movement of these little animals has driven the science of sperm study to develop and better understand the metabolic strategy undergoing sperm function, as well as to study structural components of the cell from its inner part (nucleus) to the outer one (cell membrane).

The studies in XIX and first part of XX century concentrated on fertilization and sperm motility in invertebrates, as fertilization in most of these species is external and spermatozoa could be observed easily. In 1919, in his famous book “Problems of fertilization”, Lilie reported that spermatozoa are provided with all the energy sources from their gonadal development, and that they are completely unable to find some nutrients to support motility from the external environment. To that time, this was a reasonable assessment, even if the subsequent studies focused on sperm cell physiology have deepened these themes and contributed to form a more scientific approach.

The first studies on sperm respiration were carried out in 1933 by Redenz and they were subsequently followed, in 1941, by those by Lardy and Philips on bull spermatozoa. They demonstrated that bull sperm cells can survive without seminal plasma but only in presence of sugars, and that the main energy source is the anaerobic glycolisis. In the same years, at the University of Pennsylvania, in the bacteriology unit, Zittle and co-workers (1942) performed the first studies on flagellar movements of spermatozoa, that were at that time still thought to be bacterial-like organisms. They demonstrated a cytochrome oxidase activity in epididymal bull spermatozoa, as well as an active glucose metabolism and oxygen consumption, but their studies didn’t meet the interest of the scientific community. In 1945 Lardy and coworkers deepened the studies on bull sperm metabolism, demonstrating an active Krebs cycle metabolism in these cells, increased by bicarbonate and reduced by cianure. Also ram sperm studies confirmed the presence of a functioning oxidative

phosphorylation metabolism, accompanied by the anaerobic metabolism (Lardy, 1945b).

McLeod (1941-1943) performed, in the same period, the first studies on human sperm metabolism, demonstrating their ability to use and metabolize sugars, even if he couldn't directly discriminate the activity of cytochrome oxidase, due to the low density of the samples, if compared with bull, ram and boar ones. Mann (1946) demonstrated that the main energy source for human spermatozoa is represented by fructose and later studies (Albers and co-workers, 1961) showed that the various mammalian spermatozoa present very different glucolitic/fructolitic rates.

Since that moment sperm cells have been considered as a morphological, structural and functional unit and no distinctions were made on the basis of the various compartments. Fawcett in 1957 initiated the morphological studies with the electron microscopy and gave important inputs to the knowledge of the tail structures; in 1965 Mhori and coworkers set up a good method to isolate pure mid-pieces, that lasted in studies focused on oxidative mitochondrial metabolism.

There are a lot of studies carried out later on sperm metabolism, which lead to a better knowledge of this subject, and could be found in the interesting review by Ford (2006) and Storey (2008).

When talking about sperm cell function, as movement and fertilization, we cannot forget the work by Yanagimachi, who focused on fertilization in mammals, after he began to work with two scientists who had previously discovered in vivo sperm capacitation (Chang and Noyes).

His work led him to describe in vitro capacitation process, as well as to define hyperactivated motility, with all that goes with it such as Calcium influxes, protein phosphorylation, different energy metabolism and subsequent acrosome reaction.

The development of knowledge and application of in vitro capacitation and fertilization was growing in the subsequent years, permitting a spread of the use of reproductive biotechnology in human and veterinary medicine, as well as the development of new research branches as germ cell research and cloning (for a review see Yanagimachi, 2009).

Sperm cells

Spermatozoa are the male gametes and they are highly specialized: in fact they have the aim to transport the male genetic material through the female genital tract to the other gamete, the oocyte. To do this, they undergo a maturation that starts in the testis and ends in the female genital tract, where they reach the ability to fertilize the oocyte.

Sperm Morphology

Sperm cell anatomy is peculiar for every mammalian species and is different from all the other cells of the animal body; there are anyway some common characteristics in all mammalian species. The spermatozoon is constituted by a head, a connecting piece, also called neck, and the tail, which is divided into three more parts: the midpiece, where we find the mitochondrial sheath, the principal piece, where there are the motility structures and the end piece. In the sperm head we can also distinguish two different anatomical parts: the nucleus and the acrosome (Fig. 1). Sperm cell nucleus is constituted by the haploid genomic male material and peculiar proteins binding DNA, which are histones and protamines. Histones are the typical chromatin-binding proteins of mammalian cells, but in spermatozoa they are replaced in a large way by protamines, that render sperm nucleus structure more stable and less active (Braun, 2001). The acrosome is an exocytotic vesicle deriving from Golgi apparatus and containing some important enzymes such as hyaluronidase and acrosine, which are hydrolytic enzymes fundamental to achieve zona pellucida penetration. This vesicle is rounded by a peculiar membrane, the acrosomal membrane, which is distinguished into the inner acrosomal membrane, that is nearer to the nucleus, and the outer acrosomal membrane, or external, which is just below the plasma membrane.

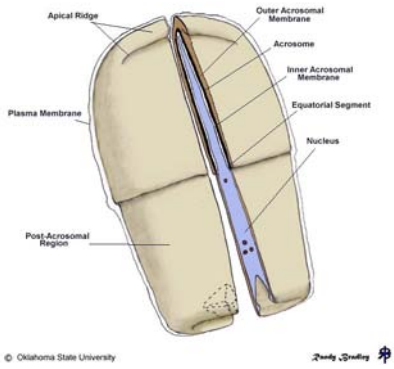

Fig. 1 The spermatozoon head (*from Pathways to Pregnancy and Parturition- Senger P. L., 2003).*

Spermatozoa tail (Fig. 2) is the main movement organ of the cell. In the neck we find the centrioluses deriving from the paternal germinal cells, which have the function of connecting the head with the other parts of the tail. The connection with the head is achieved by a dense fibrous material that binds directly to the nucleus envelope and condensed cytoplasmic material. On the other side, the segment structure changes and the first structures dedicated to movement appears. In fact, the axoneme begins in this point of the tail and goes through the whole length of this structure. The axoneme is composed of 2 α and β tubuline microtubules surrounded by 9 couples of microtubules; in the midpiece the axoneme is surrounded by 9 outer dense fibers, and in the outer part we find the mitochondrial sheath (Eddy et al., 2003). Here about 100 mitochondria are localized (Hallap et al., 2005) and they are the unique source of aerobic energy in the sperm cell through the Krebs cycle (Peña et al., 2009).

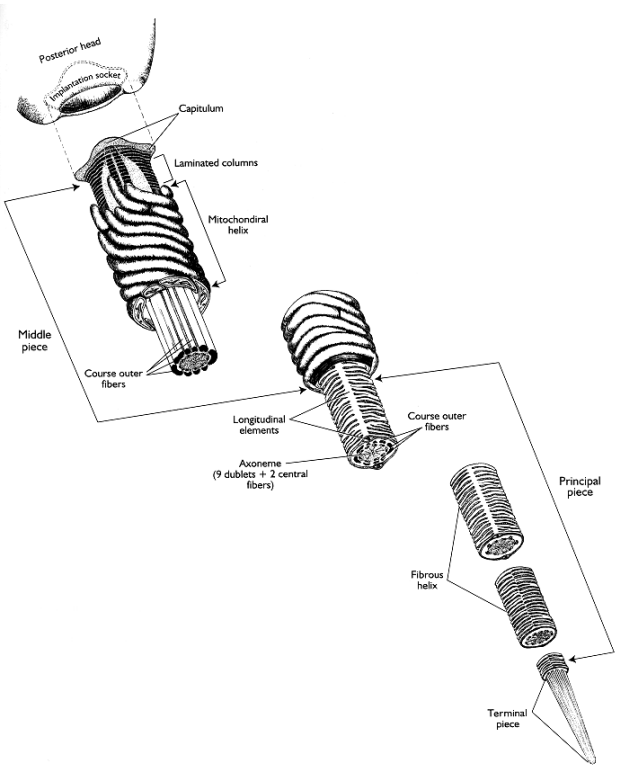

Fig. 2 The spermatozoon tail (*from Pathways to Pregnancy and Parturition- Senger P. L., 2003).*

Following the midpiece we find the principal piece, and the two structures are separated by the annular ring, also known as Jensen ring. The main part of principal piece is composed of the continuation of the axoneme, rounded by 7 outer dense fibers and by a specialized cytoskeletal structure, known as fibrous sheath (Eddy et al., 2003; Kim. et al., 2007), whose function has been identified as a protection for the axoneme, as a scaffold for enzymes involved in signal transduction and as an anchoring structure for glycolitic enzymes (Eddy et al., 2003; Kim et al., 2007). The end of the tail, named endpiece, presents only the 9 + 2 microtubules couples that are directly covered by the plasma membrane.

Sperm maturation

Sperm maturation is the process which spermatozoa undergo to reach their final structure and functionality (França et al., 2005). The maturation itself takes place in two distinct parts of the testicle, seminiferous epithelium and epididymis.

In the testis we find spermatogonia, the primordial cells that undergo cellular division to create a reserve of male gametes. Two subtypes of spermatogonia have been individuated: spermatogonia A and B, even if some intermediate forms have been discovered in mouse and pig (França et al., 2005)(Fig. 3).

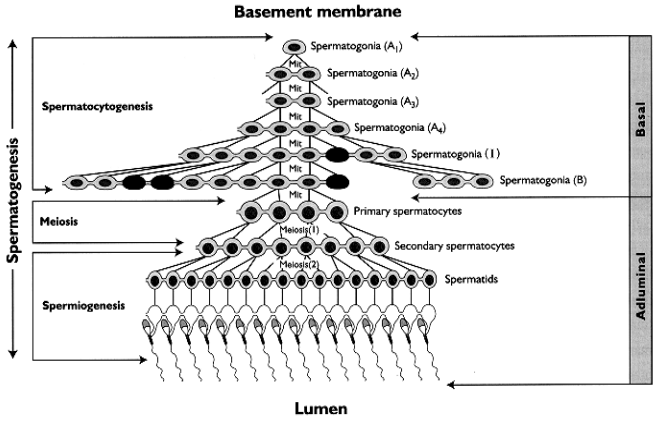

Fig. 3 Typical sequence of spermatogenesis in mammals (*from Pathways to Pregnancy and Parturition-Senger P. L., 2003).*

Spermatogonia A undergo several mitotic division, forming some intermediate cell lines (Fig. 3)

After spermatogonia B undergo a mitotic division, they form the primary spermatocytes, that undergo the first meiotic division forming the secondary spermatocytes. These ones too undergo a meiotic division, the second, that hesitates in the formation of the spermatid (Kretse and Kerr, 2008). This kind of cells still have a round form, but their genetic material is haploid. In this first phase of spermatogenesis the most important aim is to divide the genetic material of the cells, while the rearrangement of this material and the expulsion of the cytoplasm take place in a second phase called spermiogenesis.

During this process, 3 main things happen: the formation of the acrosomal vesicle from Golgi apparatus, the rearrangement of the nuclear content and the formation of the tail that also consists in the expulsion of the cytoplasm (Kretse and Kerr, 2008).

The acrosome formation consists in the transition of a part of Golgi apparatus to a pole of the spermatid, with a contextual migration of some other intracytoplasmic vacuoles containing hydrolytic enzymes. In the same time mitochondria migrate to the neo-formed axoneme, still rounded by a quantity of cytoplasm, while in the

principal piece the fibrous sheath surrounds the axoneme and changes its structure in a most dense one. Finally, the nucleus undergoes very deep changes in its structure, as there is a substitution of the main chromatin-binding protein, histones, with another protein that will be the most important one in sperm cells, protamine. These proteins make the genomic material more condensate and resistant to DNAse action, and the process ends in a reorganization of the nuclear material that renders the nuclear shape and volume peculiar for each species, as protamines are species specific and each species presents a different grade of nuclear condensation (Kretse and Kerr, 2008).

Maturation process is regulated and controlled by hypothalamus-pituitary axis and its secretion of gonadotropins as well as testosterone-estrogens by Leydig or Sertoli cells.

At this point of their lives, spermatozoa reach the end of the seminiferous epithelium and are transferred into the lumen to reach the epididymis.

The epididymis is a long tubule, constituted of three parts, head, body and tail, and in each one of these sperm cells continue their maturation, especially for what concerns the expulsion of cytoplasm, nuclear condensation, acrosome organization and membrane surface re-arrangement (França et al., 2005).

Nuclear DNA undergoes a more deep condensation, achieved by the formation of disulphide bonds, as well as it happens in the fibrous sheath (Yanagimachi, 2008).

Sperm cells transport along epididymis is achieved by smooth muscles of the tubule wall, that permit the fluid and cells to proceed. This transport is very active in the first two parts of the epididymis, while in the tail spermatozoa are stored and concentrated (França et al., 2005).

During this travel the spermatozoon loses the last part of its cytoplasm: in fact, when sperm cells arrive to the epididymis head they still have a cytoplasmic droplet near the connecting piece (proximal cytoplasmic droplet); passing through the epididymis the droplet is moved to the end of the midpiece (distal droplet) and at the end it is expulsed. The presence of a cytoplasmic droplet is a typical feature of epididymal sperm cells and could be considered as an immaturity characteristic of ejaculated spermatozoa (Harrison and Gadella, 2005).

The epididymal fluid plays an important role not only in sperm transport, but also as a fundamental component in sperm maturation and survival: in fact it contains some important factors, secreted by the epithelial cells, that adhere to the sperm surface

avoiding a premature capacitation and/or acrosome reaction (Vadnais et al., 2007). In particular these factors are proteins such as the 25 kDa protein, anti-agglutinin, secreted by the porcine epididymal duct of the corpus epididymis that subsequently binds to the acrosome of spermatozoon (Harayama et al. 1999). Another example is Crisp-1, a glycoprotein secreted by rat epididymal tail where it associates with the spermatozoon membrane (Xu et al. 1997); it is present also in man (Dacheux et al. 2006).

The secretion and adherence of protein goes on in the seminal plasma too.

Depending on the species, the spermatozoon acquires another very important feature in different parts of the epididymis: motility. In fact, testicular spermatozoa are normally less or no motile, while spermatozoa collected from the epididymal tail are always motile (Yanagimachi, 2008).

Capacitation

After the maturation process a spermatozoon is still unable to fertilize an oocyte, because it has to undergo a series of processes known under the name of “capacitation”.

Capacitation has been discovered in 1951 by two different researchers, Chang and Austin, but the major studies began in the ‘60s and are due to Yanagimachi (2009).

Ejaculated spermatozoa have to go through the whole female genital tract (FGT) to encounter the oocyte and during this passage they get capacitated to fertilize the egg (Rodriguez-Martinez, 2007).

Spermatozoa transport through the FGT is predominantly a passive process, accomplished by uterus movements and contractions (Brüssow et al., 2008), during which sperm cells lose the “uncapacitating coat”, until they reach the sperm reservoir, a particular structure near the utero-tubal junction (Rodriguez-Martinez, 2007; Brüssow et al., 2008). In this part of the uterus spermatozoa are kept protected from phagocytosis and the capacitation process is stopped, as soon as they are released and reach the tubal environment, that seems to play an important role in “in vivo” capacitation (Rodriguez-Martinez, 2007). In particular the tubal fluid composition seem to be central in promoting sperm capacitation, especially for the basic pH it reaches due to bicarbonate ion secretion.

The modifications the sperm cell undergoes during capacitation involve membrane changes in fluidity and composition, activation of tyrosine phosphorylation, calcium flux augmentation, activation of the hyperactivated motility pattern, changes in the metabolic state of the cell.

It is noteworthy that the removal of all the non-capacitating (above mentioned) agents the spermatozoon is enriched during its maturation in epididymis, is concretely an important membrane change that occurs and starts capacitation.

As for membrane changes, it has been suggested that cholesterol could play a very central role: in facts one of the molecules of which sperm plasma membrane is enriched during its passage into the epididymal lumen is cholesterol, and it is one of the molecules that are removed from its surface under the action of albumins and in particular BSA “in vitro” (Flesh and Gadella, 2000; Gadella, 2008). In this way we can identify cholesterol as a stabilizing factor of the membrane that, being removed, permits the lipidic bilayer to flow more freely as well as some external proteins to

move through the membrane (Yanagimachi, 2008). It is furthermore reported that capacitation, in species in which the cholesterol content of the plasma membrane is higher, occurs later than other species (Flesh and Gadella, 2000). The cholesterol efflux seems however to be a consequence of the activation of protein kinases (in particular PKA, via bicarbonate adenilate cyclase activation), and not to be the cause of this process (Harrison and Gadella, 2005).

The membrane structure of sperm cells is organized in microdomain of phospholipids, normally kept in a static and regulated manner (Harrison and Gadella, 2005); during capacitation, and under the action of bicarbonate, these microdomains are “scrambled” by a series of enzymes (regulated by phospho/dephosphorylation) called scramblases. This process seems to be of great importance in permitting cholesterol efflux from the plasma membrane, as well as in rendering it “fusogenic”, less rigid and ready to undergo the acrosome reaction (Harrison and Gadella, 2005).

HCO_3^- ion is fundamental in capacitation, in particular for what concerns cell activation: in fact, it is stated that in the epididymis tail the concentration of bicarbonate ion is lower than in normal serum (Flesh and Gadella, 2000; Harrison, 2004), but when spermatozoa are ejaculated in the FGT, they encounter higher concentration of both CO_2 and bicarbonate. The action of this ion is to initiate the phosphorylation of some proteins (more of which still unknown) by augmenting the activity of soluble adenilate cyclase, with an increase in cAMP and consequent activation of Protein Kinases (Fig. 4) (Flesh and Gadella, 2000; Harrison and Gadella, 2005).

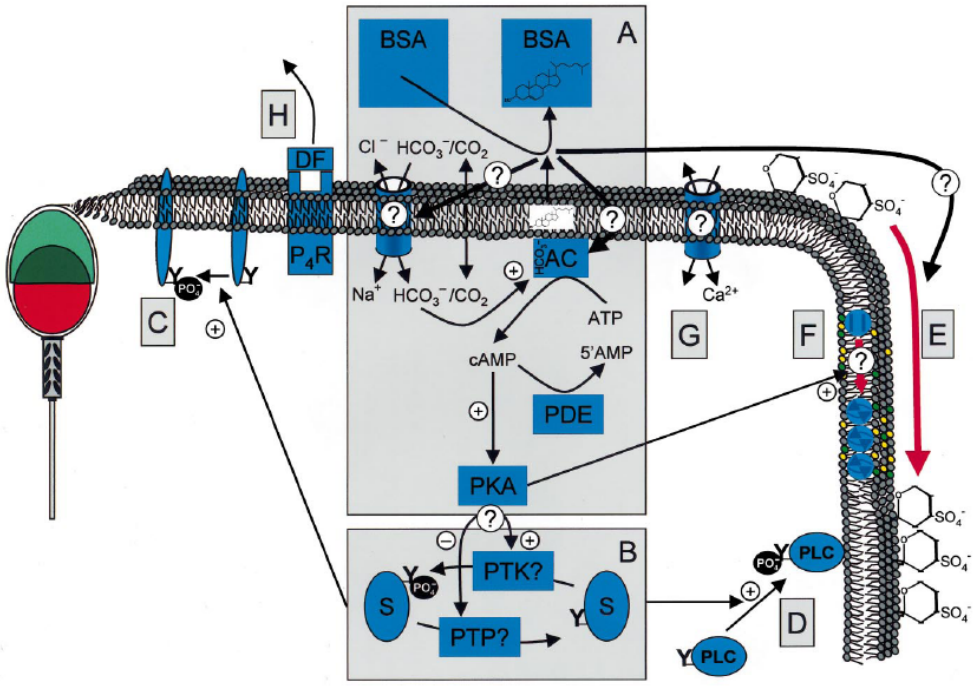

Fig. 4. A representation of capacitation phenomena (*Flesh and Gadella, 2000*). The role of BSA and HCO_3^- are stressed in this picture in activating internal mechanisms.

The sperm plasma membrane contains a variety of Ca^{++} channels: voltage dependent, Ca^{++}-ATPase, Na/ Ca^{++} exchanger and probably others (Flesh and Gadella, 2000). Capacitation, as well as acrosome reaction, is dependent on the influx of calcium into the cell, even if it is not yet well established how this calcium passes through the different regions of the cell. It is well known that calcium influx induces an activation of adenilate cyclase, with a subsequent activation of protein kinase A via cAMP, that reinforces the process of capacitation (Flesh and Gadella, 2000; Visconti, 2009). In addition, calcium is very important in membrane hyperpolarization and inner cell pH augmentation during capacitation (Vadnais et al., 2007).

Hyperactivated motility is another typical feature of capacitated sperm cells and consists in a more rapid and also non-linear movement of the cell. This kind of movement has been hypothesized and demonstrated to permit spermatozoa to penetrate the mucus barrier they encounter in the tubal lumen, as well as in trespassing cumulus oophorus cells (Suarez and Ho, 2003).

To better understand the importance and relevance of hyperactivated motility, it is helpful to remember that sperm cells acquire the capacity of moving only after their

testicular maturation, when they rich the epididymis; this kind of motility is the so called activated motility (Yanagimachi, 2008) and its principal characteristic is to be almost linear or at least to proceed in a straight trajectory. Hyperactivated sperm cells swim in a very different manner, as tails beat in a most asymmetrical manner, forming larger curves (Suarez and Ho, 2003) and resulting in a more disordered trajectory.

Hyperactivated motility is regulated by many factors, the most important of which is Ca^{++} availability. As stated above, Ca^{++} can enter the cell via the numerous Ca^{++} channels present in the membrane of the spermatozoon (Flesh and Gadella, 2000).

It is also stated that the activity of these channels can't provide the whole amount of Ca^{++} needed for the activation of the cell, so the intracellular reserve of Ca^{++} becomes of primary importance. Ca^{++} storage entering in the activation of hyperactivated motility is that found in the neck of the sperm cell, and in particular in the redundant nuclear envelope where the centrioluses anchor (Suarez and Ho, 2003; Suarez, 2008). Influx of Ca^{++} induces an activation of the adenilate cyclase and a subsequent phosphorylation in tyrosine and serine residues of the tail proteins (Suarez, 2008).

In "in vivo" trials it was tried to explain the possible role of some molecules (such as progesterone) as well as chemotactic stimuli in controlling hyperactivated motility, and relating this event to capacitation (for a review see Suarez, 2008), but the precise mechanisms are not yet fully understood.

As mentioned above, an important event occurring during capacitation is protein phosphorylation, and particularly protein tyrosine phosphorylation. This process is finely controlled: the activation of the sperm specific Adenilate Cyclase (AC) via various stimuli (Flesh and Gadella, 2000; Urner and Sakkas, 2003; Harrison and Gadella, 2005; Suarez, 2008) induces an accumulation of cAMP that initiates the activity of some protein kinases, in particular PKA, that initiates the threonine, serine, tyrosine phosphorylation of some proteins. PKA activation drives to activate PKC, that reinforces calcium entrance flux. At the same time some parallel pathways are activated, such as the AKAP and ERK ones (see Fig. 5).

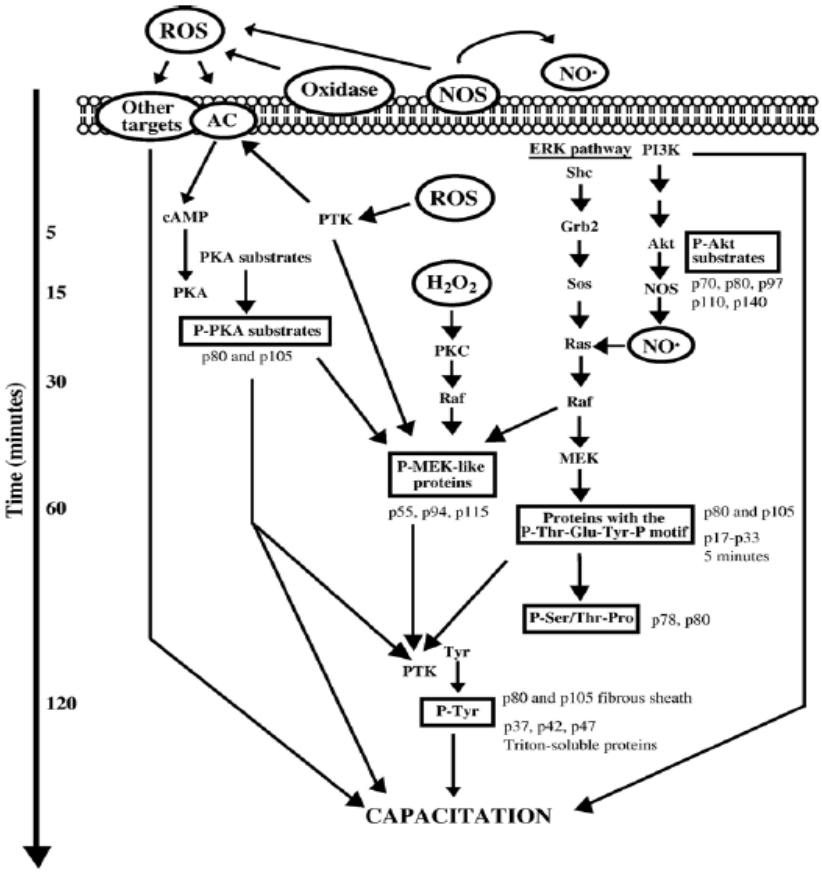

Fig. 5 Intracellular pathways in capacitation (de *Lamirande and O'Flaherty, 2008*). The role of Protein kinases is described in this picture, as well as the activity of ROS.

We should not think of these events as separated or independent, because it is well demonstrated that they are interdependent and surely it's possible to individuate crosstalks between these pathways (Breitbart and Naor, 1999; Breitbart et al., 2006). During capacitation there is also a translation of PLC (phospholipase C) to the plasma membrane, via the activation of some substrates of PKA, that is an important passage for the initiation of the subsequent acrosome reaction (Flesh and Gadella, 2000).

Another aspect that is fundamental in reaching the capacitated status is production of reactive oxygen species (ROS): the more representative agents are H_2O_2, NO^-, OH^- . These molecules are usually retained negative in cell survivor and function, as they affect membrane integrity and nuclear function. If produced in small quantities they have been discovered to be very important in capacitation (de Lamirande and Gagnon, 1995; Dröge, 2001; de Lamirande and O'Flaherty, 2008). As stated in

Figure 5, ROS enter the activation of many pathways regarding protein tyrosine phosphorylation and contribute to the enhance of capacitation.

Acrosome reaction

Acrosome reaction is the last pre-fusion phase of the living spermatozoon. As some sperm cells reach the ovum (a significantly lower number than that ejaculated, see Brüssow et al., 2008), they are still unable to fertilize it, because mammalian ova are surrounded by a glycoproteic coat, zona pellucida (Yanagimachi, 2008). To penetrate this coat, a sperm cell must adhere it and then go through an exocytotic process, known as acrosome reaction. The main enhancer of the acrosome reaction is zona pellucida, even if also cumulus oophorus matrix could play some role (Yanagimachi, 2008).

It is well stated that ZP 3 (or C) and progesterone, via its non-genomic receptor (Breitbart and Sungin, 1997; Flesh and Gadella, 2000) (Fig. 6) are the most important inductors of acrosome reaction. Sperm surface is provided with adequate Zona Binding Proteins, that are exposed during the capacitating process (Flesh and Gadella, 2000; Gadella, 2008b), and activate these receptors via tyrosine phosphorylation and aggregation. At the same time progesterone can bind to its non-genomic receptor and the consequence of these two events are an increase of the intracellular pH via G protein and a depolarization of plasma membrane. As a result, Ca^{++} channels open and permit a massive influx of Ca^{++} ions that permit the activation of the phospholipase C (PLC), already translocated to the plasma membrane during capacitation. Activated PLC converts phosphor-inositol-di-phosphate (PIP_2) to diacilglycerol (DAG) and Inositol-3-phosphate (IP_3). At the same time, the high levels of calcium activate cAMP/PKA pathway, leading to a major release of calcium from the acrosomal storage; this Ca^{++} concentration increase leads to the activation of phospholipase A2 (PLA_2) that acts in degradating phosphocoline (PC) to lysophosphaditilcholine (LPC) and free fatty acids. All this secondary products activate PKC (Flesh and Gadella, 2000), as well as Ca^{++}concentration augmentation and activation of PKA (Breitbart and Spungin, 1997; Abou-Haila and Tulsiani, 2009; Breitbart et al., 2009). PKC migrates toward the membrane and initiate fusion process, that will lead to the final hexocytosis.

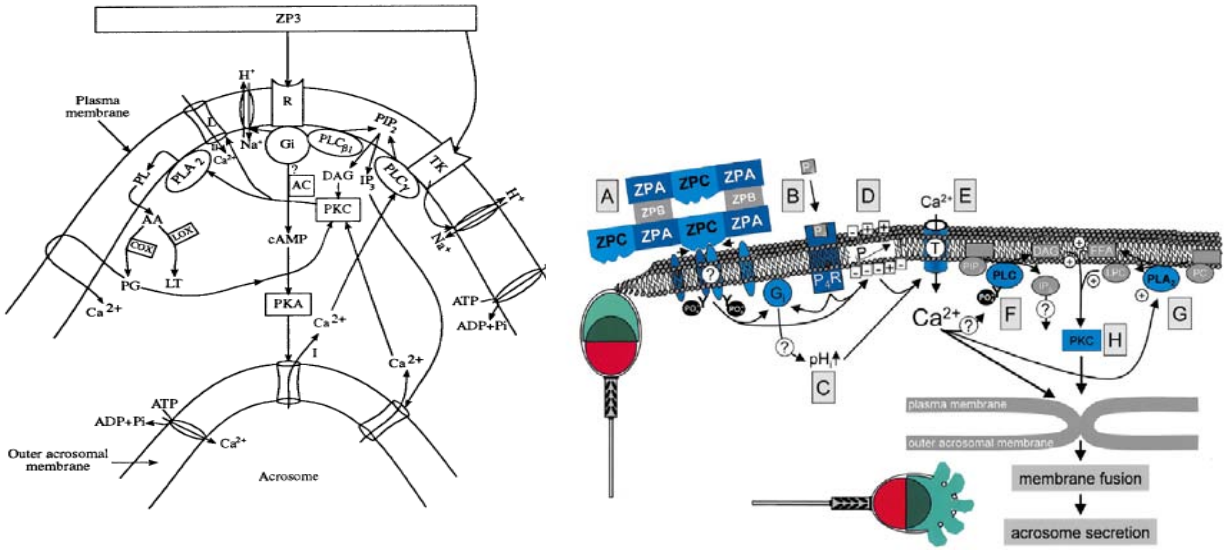

Fig. 6. Acrosome reaction mechanisms (Breitbart and Spungin, 1997; Flesh and Gadella, 2000). The importance of ZP and Ca^{++} is stressed in this figure, as well as the various intracellular mechanisms leading to acrosome reaction.

In studing acrosome reaction a particular attention should be given to actin cytoskeleton changes and especially to actin polymerization during capacitation and its subsequent depolimerization just before acrosome reaction (Breitbart et al., 2005). It is stated that actin is present in various mammalian species sperm head and tail, thus suggesting that it could be involved in sperm motility as well as other functions as capacitation and acrosome reaction (Breitbart et al., 2005). It is still unclear if it is in the monomeric form (G actin) or in the polimerizated or filamentous form (F actin), and the presence of the numerous actin-bound proteins (that are necessary for actin polymerization/depolymerization) in sperm cells is a proof that the two forms are present in spermatozoa (Breitbart et al., 2005). Some Authors described a polymerization of actin during capacitation in various mammalian species (Castellani-Cresa et al., 1993; Cabello-Aguero et al., 2003; Brener et al., 2003), and a depolymerization of F actin right before acrosome reaction (Brener et al., 2003).
This could explain the actin role, as its polymerization is necessary to reach the capacitated status as well as the fertilizing ability in many mammalian species (Rogers et al., 1989; Castellani Cresa et al., 1993; Brener et al., 2003; Cohen et al., 2004), and its breakdown is necessary to obtain an acrosome reaction (Spungin et al, 1995; Breitbart et al., 2005) (Fig.7).

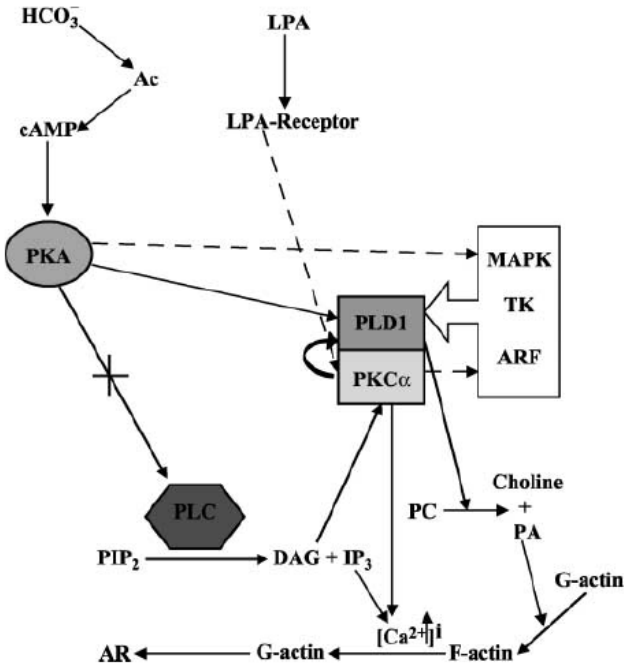

Fig.7 Actin polymerization in capacitation and acrosome reaction (Breitbart et al., 2005). The mechanism of polymerization during capacitation and depolymerization during acrosome reaction of the actin cytoskeleton is described in this figure

.

Sperm metabolism

When talking about sperm metabolism, we have to keep clear that the main goal of sperm cells is taking male haploid DNA to the female one, and that, to do this, they have to move across the female reproductive trait. Therefore, movement is the main function of sperm cells and the main aim of energy obtainment. Anyway, we should not forget that sperm cells undergo some functional changes that permit them to acquire the fertilizing ability, such as capacitation and acrosome reaction; during this functional moments energy requirements are not only dedicated to movements, but also in activating internal cell functional pathways such as protein tyrosine phosphorylation, calcium channel activation, hyperactivated motility and acrosome reaction (Flesh and Gadella, 2000).

Sperm cells need energy for moving, as it is the main goal of their living condition. They acquire the capacity of moving after the epididymal maturation (Yanagimachi, 2008), as in testis sperm cells are actually non-motile. Active movements are necessary to pass through the female genital tract, even if in most of this journey the most important “transport” of the male genomic material is performed by the female genital tract itself, by movements of the smooth muscles of the uterus and tuba (Rodriguez-Martinez, 2007; Brüssow et al., 2008).

Sperm cells use sugars as an energy source: they can use hexoses, such as glucose, mannose, fructose, but they can also use some other sources, such as lactate and citrate. The two metabolic pathways involved in sperm energy obtainment are anaerobic glycolysis and oxidative phosphorylation. In fact, as already stated, spermatozoa have a mitochondrial sheath in the midpiece, where the oxidative processes may take place, and the glycolitic enzymes in the principal piece of the tail, connected to the fibrous sheath (Eddy et al., 2003; Ford, 2006).

There are different opinions whether glycolysis or oxidative phosphorylation is the major source of energy, in the form of ATP, for sperm cells. In particular Miki and co-workers (2004; 2007) demonstrated the predominant role of glycolysis: in fact they produced genetically modified mice, lacking the gene of glyceraldehyde-3-phosphate, that is a very important enzyme in the glycolitic chain. These mice’s spermatozoa have an ATP production 90% decreased than normal mice, and their motility is very much decreased. These data support the hypothesis that the only way sperm cell use to get energy from a sugar substrate is the glycolitic one, while ATP production

from oxidative phosphorylation is not indispensable for motility (Miki, 2007). What's more, they affirm that glucose is indispensable for capacitation in mouse, while the absence of pyruvate or lactate (that are metabolized directly in the oxidative phosphorylation cycle) do not affect capacitation. As a last proof of the prominence of glycolysis, they elaborated some trials with inhibitors of the phosphorylation chain, not affecting the movement of the cells (Miki, 2007).

On the other hand Ruiz-Pesini and coworkers (2007) retain that oxidative phosphorylation is central in sperm motility and sperm function: they report studies in which it is stated that mitochondrial function and sperm movement are associated, or that the use of mitochondrial activity enhancers results in a more relevant activation of sperm motility and fertilizing rate in human. They also report other studies demonstrating the increase of oxygen consumption related to an augmented motility pattern, as well as many studies reporting that inhibitors of phosphorylation chain hesitate in a impaired motility and fertilizing rate. Finally, when taking into account Miki and coworkers' results (2004), they state that glyceraldehydes-3-phosphate dehydrogenase (G3PD) knockout mice, used as a model to demonstrate that glycolysis is the main energy-producing pathway, are not the best to use or almost the interpretation of the results is not correct. In fact they affirm that the lack of G3PD makes the glycolitic process (that can only form 2 molecules of ATP, pyruvate and NADH for each glucose molecule) an energy dispersive process, and not an energy productive. In fact the positive balance of the entire glycolitic chain is achieved after the G3PD step, where 4 ATP molecules are produced, while prior to this passage ATP is used to phosphorylate glucose and fructose. From this point of view Ruiz-Pesini and coworkers (2007) affirm that ATP is produced in oxidative phosphorylation process and that it is used by sperm cells to metabolize glucose and to move. This fact could also explain the other results by Miki et al. (2004) that demonstrated that glucose rich medium negatively affect motility in knockout mice for G3PD: in fact, having more glucose to process, energy balance is displaced toward the ATP-consuming impaired glycolysis, and not toward motility pattern.

The presence of such different interpretations of sperm metabolism implies a great difficulty in approaching this theme: is glycolysis the main ATP source for sperm metabolism or is it oxidative phosphorylation? Probably the best approach to the argument is furnished by Ruiz-Pesini et al. (2007): they simply define glycolysis and oxidative phosphorylation as two interdependent and consequent pathways, that only

depend on the presence of a carbon and oxygen source. Being glycolysis less efficient in obtaining ATP, it is normal that sperm cells (like other cells) obtain energy from the aerobic pathway, as they have the possibility to do that with their enzymatic mitochondrial apparatus.

Another important theme regarding sperm metabolism is protein phosphorylation: this event, occurring mainly during capacitation (Flesh and Gadella, 2000; Urner and Sakkas, 2003; Suarez, 2008), is important to activate some protein functions and has been demonstrated to be strictly related to the achievement of hyperactivated motility (Flesh and Gadella, 2000; Suarez, 2008). These are ATP consuming events that are regulated by the availability of hexoses (glucose primarily, as stated in mouse by Urner et al., 2001) and by the activity of the catabolic pathways involved in sugar metabolism (Mukai and Okuno, 2004). Tail proteins get phosphorylated as the capacitation process proceeds: in fact at the beginning of capacitation in mouse spermatozoa (Urner and Sakkas, 2003) we can find protein phosphorylation in the principal piece of the tail, and subsequently even the midpiece is involved. In general, a wide augmentation in protein phosphorylation is recognizable during capacitation, and this could be thought as a major request to produce energy from the metabolic apparatus.

When talking about capacitation and hyperactivated motility, we should remember that the engine of the spermatozoon is the tail, and precisely the principal piece. At the same time we should take into account that midpiece, where mitochondria are set, is divided from the principal piece by the distal annular ring, and this, as the tail length, could represent a problem in delivering energetic substrates to the dinein-tubuline engine. This aspect has been described by Ford (2006) and it is stated that diffusion of ATP is possible thanks to sperm movement itself, as well as to some protein delivering ATP to other sites (Ford, 2006).

The compartmentalization of the spermatozoon is important in understanding some metabolic pathways: in fact, it is demonstrated that glycolitic enzymes are located in the tail of the cell (Eddy et al., 2003; Krifalusi et al., 2004), as well as some other proteins involved in cell signaling as AKAP4, AKAP3, rhophilin and ropporin (Eddy et al., 2003). Glycolitic enzyme products are taken to the mitochondria or secreted in the external medium (as lactate), but ATP produced in the tail should, at least in part, remain in this site to permit movement and phosphorylation of some proteins (Urner and Sakkas, 2003). What's more, in sperm head there are no glycolitic enzymes,

except for hexose kinase 1, but it has been reported that glucose may play a role in gamete fusion, and in particular that NADPH is very important during this functional moment (Urner and Sakkas, 2005). In this item the pentose phosphate pathway is another important metabolic strategy implied in sperm function: in fact it is important to keep the reductive potential of the cell, as well as to fertilize the egg (almost in mouse). This metabolic pathway could take place in the head or in sperm tail (Urner and Sakkas, 2003; 2005) (See Fig. 8).

The possible role of pentose phosphate pathway in the midpiece is not yet well defined, as it is possible that the isocitrate and malate pathways take place in the mitochondria (Urner and Sakkas, 2003).

During capacitation and, preeminently, acrosome reaction there is an activation of Ca^{++} channels and some of them are ATP-consuming channels (Flesh and Gadella, 2000; Harrison and Gadella, 2005; Miki, 2007). Therefore, during capacitation and acrosome reaction there is another fount of ATP consumption, that contributes to the rise in overall metabolism of sperm cells. ATP is required to undergo acrosome reaction (Miki, 2007), but it is still unknown how ATP does reach the head of the cell; Miki (2007) proposed that it could be generated by oxidative phosphorylation in mitochondria, and could be transposed to the cell head more easily than what could happen with glycolysis. The compartmentalization of sperm cell and the presence of pentose phosphate pathway in the head (Urner and Sakkas, 2003, 2005); could be important in limiting negative effects of ROS (Williams and Ford, 2004) or in modulating their activity (Storey, 2008).

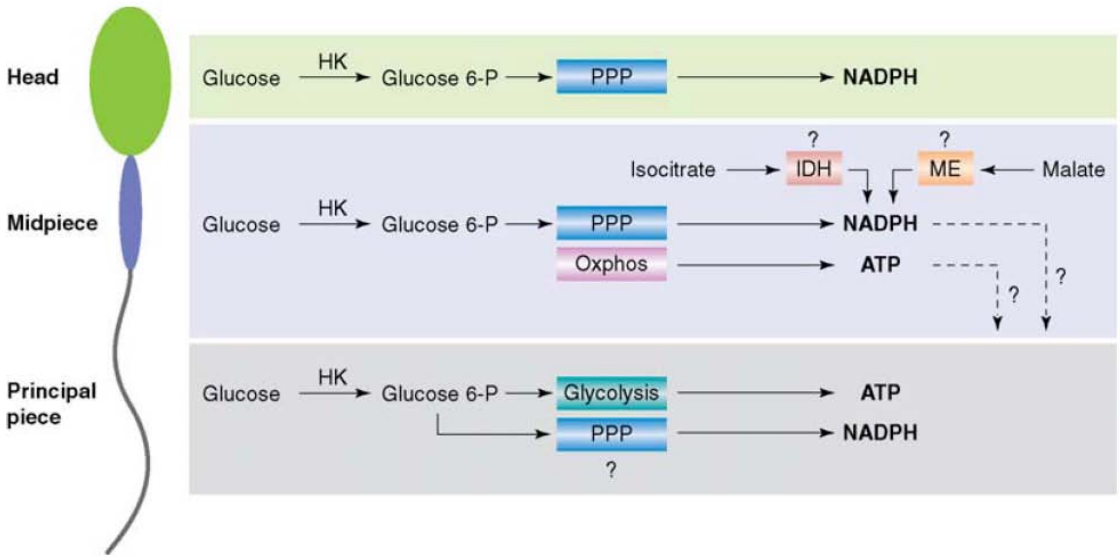

Fig. 8. Compartmentalization of sugar metabolism and protein phosphorylation in sperm cell (*Urner and Sakkas, 2003*). The figure describes the different metabolic ways implied in the distinct parts of the spermatozoon.

The approach to be taken in studying sperm metabolism should be to analyze the differences between the various species to avoid any generalization that could lead to misunderstandings.

A study in boar metabolomic demonstrated the peculiar utilization of monosaccharides by this species (Marin et al., 2003); in fact, it is stated that the glycolitic pathway is the most important in freshly ejaculated boar spermatozoa, as the production of lactate at this state is relatively high. On the other side, the pentose phosphate shunt, that is very important for the maintenance of the reducing capacity of the cells, is almost absent, in particular for what concerns the aerobic part of the reaction. The gluconeogenesis does not seem to take place in boar sperm cell, while a very little quantity of glycogen is produced. The incubation of sperm cell with lactate induced production of CO_2 that can be referred to the activation of the aerobic Krebs cycle via PDH (pyruvate dehydrogenase), even if this could represent only a 5% of the energy production. No fatty acids synthesis was demonstrated to occur in presence of glucose (Marin et al., 2003).

In this metabolic activity glucose, as well as fructose, play an important role (Jones and Connor, 2000), as they are the main substrates present in boar seminal plasma. It was stated that glycolysis is the main pathway to obtain energy for freshly ejaculated spermatozoa, and it was hypothesized that this could be the main energy reserve for tail movements (Mukai and Okuno, 2004; Medrano et al., 2006), even if others have different vision about this question (Folgero et al., 1993; Ruìz-Pesini et al., 1998) in other species. Glucose plays the central role in boar sperm metabolism, as it is the most “lactogenic” sugar, as well as the most phosphorylated one (Medrano et al., 2006). This could be explained with the relative lack of fruttokinase in boar (as well as in dog, Ballester et al., 2000), that ends in a forced passage of fructose through hexokinase, that has a lower affinity for this sugar (Medrano et al., 2006). Hexokinase itself works as a real control point in the entrance of substrates in the glycolitic pathway. Another important control point in boar sperm metabolism is Pyruvate Kinase (PK): in fact, being this enzyme ADP dependent, a high rate of ADP

(achieved in highly ATP production) down regulates its activity (Medrano et al., 2006) and doesn't permit pyruvate to enter the Krebs cycle, as it seems to happen in boar sperm. What's more, tyrosine phosphorylation is not affected by the different sugars present in the milieu, attesting the difference with other mammalian species as dog (Rigau et al., 2002).

This control role achieved by hexokinase and PK coupled with that of lactate dehydrogenase, has been referred by other Authors to the presence of two isoforms of the enzymes or, at least, to the presence of two different substrate affinity of the same enzyme (Medrano et al., 2006b); the high affinity function is carried out in different conditions, as low concentrations of substrate and the original source of the substrate itself. More, double affinity enzymes have been found in dog sperm (Fernandez-Novell et al., 2004), thus indicating this could be a feature of mammalian sperm cells.

Another important information about boar sperm metabolism is the possibility to use exogenous lactate and citrate. In facts, boar spermatozoa utilize these non-hexose sugars directly introducing them in the Krebs cycle (via LDH) or indirectly by converting citrate into lactate. The final result of this process is the obtainment of ATP and the production of CO_2 (being this last the indicator of the involvement of the aerobic part of glycolysis) (Medrano et al., 2006b). In boar, as well as in dog, enzymatic activity is more effective at low concentration of substrate (physiologic-like) than those found in normal sperm diluents.

Another different feature is found in dog spermatozoa (Ballester et al., 2000; Marin et al., 2003; Rigau et al, 2002). It is in fact well known that dog spermatozoa can obtain glycogen from monosaccharides, particularly glucose and fructose. More precisely, there is a strict regulation of glycogen synthesis by the different hexoses, as fructose strongly activates glycogen synthase, with no effect on glycogen phosphorylase, while glucose activates the glycogen synthase in a fainter manner, but it contextually down-regulates glycogen synthase activity. Another interesting difference between fructose and glucose is that the first one is more effective in activating sperm glycogen production, probably due to the higher capacity to produce a very important substrate such as glucose 6 phosphate (G-6P). Glycogen and glycogen synthase are localized in the sperm head and midpiece. It is not surprising to find this molecules in sperm head, as some hexokinase activity has been described to take place in the head, producing hexose phosphates, that are important for sperm-egg fusion

(Ballester et al., 2000). What's more, in dog spermatozoa the energy production in form of ATP is highly activated by fructose, more than glucose, as well as lactate and CO_2 production, as a consequence of the major G 6-P production enhanced by fructose (Rigau et al, 2002). In this way, even ribose phosphate production, that could be considered a pentose-phosphate-pathway activation indicator, is higher with fructose than glucose, as a consequence of the previously described glucose 6 phosphate production. Furthermore, fructose augments ATP consumption in dog sperm cell via two different ways: the first one is to activate a more rapid and linear velocity in sperm movements, and the second one is to activate a general phosphorylation in the sperm cells, involving hexoses as well as tyrosine protein residues (Rigau et al., 2002). A circling of glycolitic substrates could take place in dog spermatozoa as it was described in bull sperm cells (Hammersted and Lardy, 1983) and it exits in an activation of the cell function, even if not in a net ATP production that explains the low difference in net ATP production between glucose and fructose (Rigau et al., 2002).

For what concerns bull spermatozoa, it is stated that they can metabolize hexoses via glycolitic and oxidative pathways (Hammersted and Lardy, 1983), and that glucose is the favorite substrate to support motility and sperm function; in addiction, they stated that pyruvate is metabolized to lactate and finally used in mitochondrial oxidative phosphorylation. In the same study those Authors stated that bull ejaculated spermatozoa usually do not produce the theoretical stoichiometric ATP rate from glycolysis, as there is a strong circling between hexose phosphate derivates, such as glucose phosphate, fructose biphosphate and monophosphate, depending on the metabolites flux: the presence and activity of phosphatases as control points in the metabolic processes of bull sperm is affirmed.

On the other hand, epididymal bull spermatozoa demonstrated the capacity to produce lactate and to metabolize it through Krebs cycle to obtain ATP, obtaining a theoretical production rate, thus indicating that accessory sex glands provide non-activating molecules to the sperm. This observation strongly relates to the fact that bull spermatozoa capacitation is inhibited by glucose (Storey, 2008). In the female genital tract the low level of glucose (Storey, 2008) permits a regular achievement of the capacitated state by spermatozoa, and this fact has also been demonstrated in vitro: glucose added to capacitation media had the effect to reduce the capacitation rate by lactate production (Galatino-Homer et al., 2004). In fact it is stated that bull

sperm can reach the capacitation state in media containing Ca^{++} and heparin, and that, after capacitation, internal cell pH is higher (Storey, 2008). This rise in intracytoplasmic pH is achieved by the augmentation of cAMP via heparin receptor or activation of PKA pathway. The hexoses circling could represent a regulatory pathway of these events.

Data concerning horse spermatozoa are lacking: in fact, all the studies that have been conducted till now are more interested in checking performance and complexion of horses, more than reproductive performance. The lack of studies on reproduction in horses is more and more deep in spermatology and in particular in sperm metabolism.

It has been stated that horse spermatozoa, like other mammalian sperm cells, have the enzymatic pool for glycolysis (Westhoff and Kamp, 1997), in particular the presence of glyceraldehydes-3-phosphate dehydrogenase, and that glycolysis products are delivered to mitochondria to be processed under oxidative phosphorylation. Mitochondrial function has been assessed and demonstrated using fluorescent probes (Gravance et al., 2000). These proofs were made on fresh ejaculated stallion semen, so that it is likely predictable that both glycolysis and oxidative phosphorylation participate in stallion sperm motility and overall metabolism.

Some works by Mann (1974; 1975) report a good availability of glucose in stallion seminal plasma, a lack of fructose, and the presence of inositol and citrate, thus suggesting an ability to produce energy from hexoses.

It is not yet clear wherever glycolysis or oxidative phosphorylation plays the main role in freshly ejaculated spermatozoa, even if it is likely that glycolysis is mainly involved in furnishing energy for the high mobility of these spermatozoa. No data are shown on energy metabolism during capacitation: recently, a strong protein tyrosine phosphorylation has been demonstrated to occur in stallion spermatozoa under capacitating condition (McPartlin et al., 2008) as well as an active control role carried out by phosphatases (Gonzalez-Fernandez et al., 2009). Recent in vitro experiments (McPartlin et al., 2008; 2009) focused on the importance of tyrosine phosphorylation and hyperactivated motility in stallion sperm capacitation as well as in fertilization, revealing a lack of information on the previous metabolic strategies used by horse spermatozoa to achieve these functions.

To conclude this argument, it is important to keep in mind that domestic mammalian species have different physiologic reproductive strategies, with a strong impact on sperm cell energy management and metabolism: as reported by Rogriduez-Gil (2006) it is very different to talk about a dog sperm cell, that could have to survive in the genital female tract for two weeks, or to talk about a bull spermatozoon, that has to reach the oocyte in a very short time (as short is the estrous period of the cow).

Therefore, it is possible to define different sperm cells "metabolic phenotypes" (Rodriguez-Gil, 2006), mainly represented by dog phenotype and boar phenotype. Dog phenotype spermatozoa have a finely controlled hexose metabolism (Ballester et al., 2000; Rigau et al., 2002; Fernandez.Novell et al., 2004) with the presence of two distinct hexokinases, with an anabolic glicogenosinthetic pathway (Albarracin et al., 2004), that play important roles in cell surviving and cell capacitation. In addition, the supposed presence of a phospho-hexoses circling (Rigau et al., 2002) is another control point in achievement of energy and its utilization and administration.

Boar spermatozoa phenotype is quite the opposite, as it is a strictly glycolitic cell (Marin et al., 2003) that hasn't an important anabolic energy management: the main interest in hexose metabolism is the obtainment of energy to support motility and cell homeostasis.

The other domestic species have their own "metabolic phenotype", but we must recognize that the main feature of mouse (Miki et al., 2004), stallion (Westhoff and Kemp, 1997) and bull (Hammersted and Lardy, 1983) freshly ejaculated spermatozoa is to have a mainly glycolytic energy management.

In different functional moments of sperm cell life, such as capacitation, acrosome reaction, sperm-oocyte fusion, the cells' requirement change inevitably (Rodriguez-Gil, 2006); the strong activation of the motility, that occurs in every mammalian sperm cell, the high rate of protein phosphorylation, that reflects a protein and functional activation of the cell, are energy consuming events, that lead to major requirements that should be supported by an augmented energy production. This subject has been partially studied only in dog spermatozoa (Fernandez-Novell et al., 2004), while there is a general lack of studies for the other species, in particular on the possible activation (or hyper-activation) of the oxidative phosphorylation pattern in the mitochondria.

Another very important argument that is not so well studied and deepened is the physiological composition and role of seminal plasma: the last studies on "metabolic"

composition of seminal plasma in mammalian species have been made in the ‘80s (Gardner and Hafez, 2008) and now there is a clear discrepancy between these studies and the “metabolomic” ones. There are certainly differences between bull seminal plasma, rich in fructose and citric acid (like ram one) and horse or pig ones, that are extremely poor in these substrates; in the same manner, the presence of other sugars such as inositol and sorbitol is not yet fully understood.

The disposability of energetic substrates in physiologic liquid for sperm cells is well known (Gardner and Hafez, 2008), but these sources are in the milieu and should be up-taken by sperm cells to be utilized.

Hexose transporters

Glucydes are polar molecules, very rich in –OH groups, that can pass through the lipidic bilayer in a very slow and inefficient manner, so that cells need to include them by carriers. The carriers presence was stated at the beginning of '50s, when some researchers started to observe the dynamics of glucose uptake in erythrocytes (Widbrand in Davson, 1964; Widdas et al., 1952), even if they didn't discover the real nature of these mechanisms. Widdas (1952), anyway, showed that the dynamic of glucose transport into erythrocytes follows the typical enzymatic saturation kinetics, applying the Michaelis-Menton law in the sugar transport of glucose in placental tissues. Anyway other authors, such as Carruthers (1990) proposed a more complex enzymatic transport for the red blood cell, that became the target cell to study glucose transport.

Only in 1971 Jung and coworkers demonstrated the localization of glucose transporters in the plasma membrane: this was an important issue, because it was demonstrated that the lipidic bilayer itself cannot transport sugars, and needs some particular molecules that possess an enzymatic-like capacity to transport sugars.

Two types of sugar transporters are known, and they correspond to two different families of transport proteins, SGLTs (sodium dependent glucose transporters) and GLUT.

SGLTs actuate an active transport of sugars, in particular glucose (Sheepers et al., 2004): in fact they are sodium-glucose co-transporters or symporters, and they spend energy to permit the passage of these molecules across plasma membrane. In particular, this family is composed by six different proteins: first SGLT 1 and 2, that are Na^+ dependent glucose transporters, SGLT3, that is a glucose sensor. Then other members of SGLT family do not transport only sugars: SGLT4 and 6, that are widely diffused inositol and vitaminic transporters, and SGLT5 that is the thyroid iodide transporter (Sheepers et al., 2004). The main characteristic these proteins share is to have a secondary α-helix structure that crosses plasma membrane 14 times, and to have both N and C-terminus in the external face of the membrane.

In particular, SGLT1 and 2, the sodium dependent transporters, are those that more fit the definition of co-transporters or symporters: in fact, the favorable concentration gradient transport of sodium ions, that creates a membrane potential, is coupled to a glucose transport across the membrane. On the other side of the cell membrane, an

ATP-dependent Na^+/K^+ pump maintains the physiological state of the membrane polarization.

SGLT1 is especially expressed in small intestine, kidneys and heart, and it is a high affinity, low capacity glucose transporter, with a Na^+/glucose ratio of 2:1 (Hediger et al., 1989; Wright et al., 1994; Wright, 2001); SGLT2, on the contrary, is a low affinity, high capacity glucose transporter with a Na^+/glucose ratio of 1:1 widely expressed in all the tissues, with a particular important role in kidney (Wright, 2001; Zhou, 2003).

SGLT3 role is not yet fully understood*:* in man it doesn't transport sugar, but it behaves as a neural sensor (Diez-Sampedro et al., 2003). It has been found in the cholinergic fibers of small intestine and in the skeletal muscle, at the neural muscle junction. In these positions it seems to work as a glucose sensor, that transmits the information about glucose concentration of the external fluid directly inducing a membrane depolarization or via a secondary effector as a G protein conjugated molecule. On the other hand, in pig it has been demonstrated that SGLT3 perfectly works as a Na^+/glucose co-transporter with a lower glucose affinity, but a higher sugar specificity (Diez-Sampedro et al., 2000; 2001).

The era of GLUTs studies began in the 1977, when GLUT1 was first isolated and purified from its native cell, the red blood cell (Kasahara and Hinkle, 1977): this transporter represents almost the 5% of all the red cells membrane, and it results to fit with the kinetic properties observed later in the entire red blood cell (Wheeler and Hinkle, 1981).

As for GLUTs' structure and localization in erythrocytes membrane, they were described firstly by Muekler and co-workers in 1985; in their work, those authors stated for the first time the genomic sequence of a glucose transporter and its hypothetical structure. They described a 55 kDa membrane protein, with 12 α-helix spanning domains, an oligosaccharide binding site, and the N and C-terminus site in the cytoplasmic domain (Fig. 9).

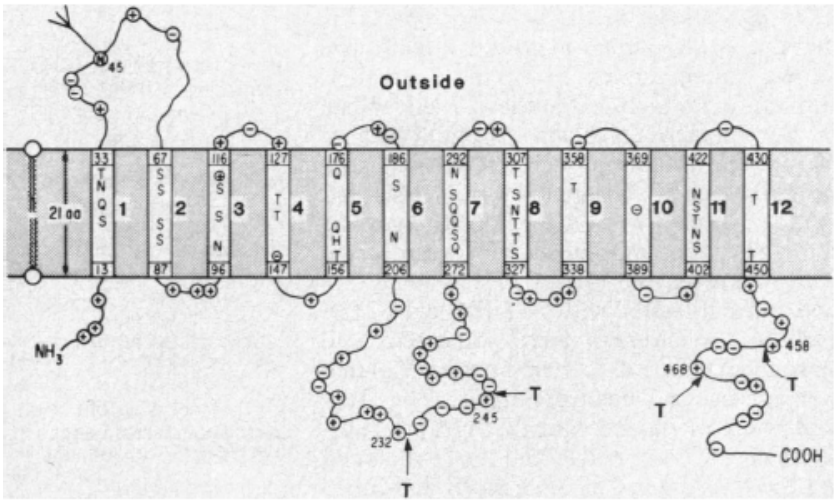

Fig. 9. GLUT 1 hypothetical structure (*Muekler et al., 1985*).

Their hypothetical structure was confirmed by Lamieux et al. (2003), who could crystallize a strictly related transporter of Drosophila.

The main feature of these transporters is that they are energy independent in their function, and this represents the main difference with SGLT family. In addition, as stated by Mueckler (1994), being these transporters passive, they can only facilitate the transition of a substrate according to the concentration degree, and this kind of transport is effective only in the case of a relative constant concentration of the substrate. This fact can explain the importance of GLUTs in mammals and other complex organisms, that are the only ones that can achieve a good regulation of hexoses concentration. Following this hypothesis, it is easy to understand how a complex organism could regulate its hexose levels in different compartments depending on the necessities of the districts, i.e. to furnish glucose to brain or muscles, or to regulate its blood concentration in post-prandial period. The mechanism of transport was also studied by other groups, and the dynamics of glucose transport was described very deeply by Carruthers (1990).

It is noteworthy that GLUTs family subdivides into 3 classes (Joost and Thorens, 2001; Sheepers et al., 2004), depending on tissue distribution, hexose specificity and structural similarity.

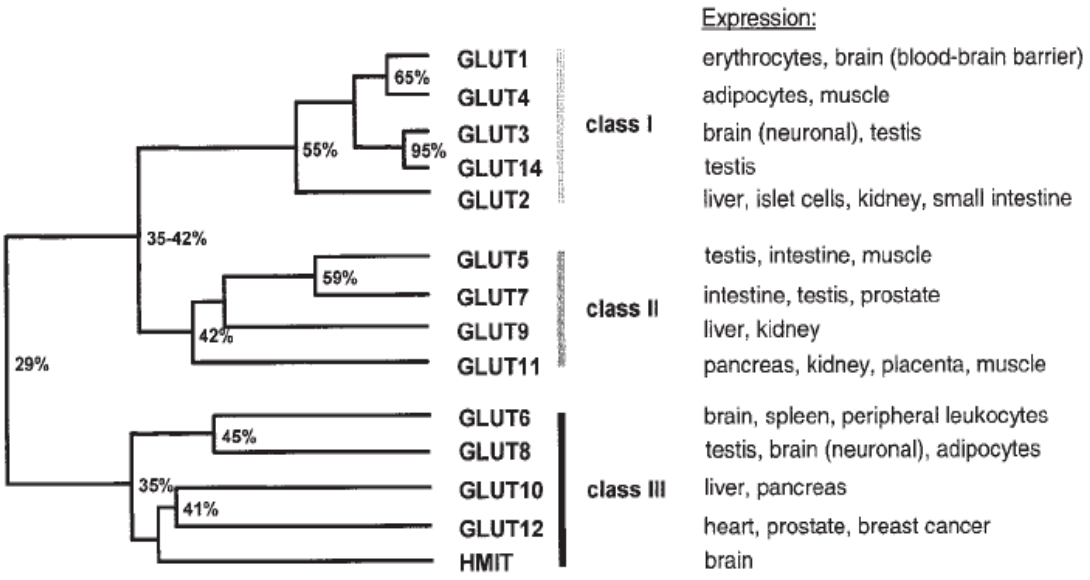

Fig. 10. GLUT family and classes division (*Sheepers et al., 2004*).

Studies carried out on the structure and genic differences into the members of the family reported by Joost and Thorens (2001) show some peculiar characteristics of GLUTs:

- 7 conserved glycine residues within the helices
- several acid and basic residues on the protein surface
- 2 conserved tryptophan residues
- 2 conserved tyrosine residues

The class I members are the most known and studied transporters: GLUTs 1, 2, 3 and 4, with the recently discovered GLUT 14. They all have in common a similar structure and tissue distribution, as well as the hexose affinity.

GLUT 1 is, as reported above, the first discovered GLUT family member and probably the most studied. It is also called red cell, brain or Hep2b glucose transporter, as these tissues or cell cultures are the most studied. For what concerns its kinetics, a lot of studies have been carried out (see for a review Muekler, 1994): the transporter has been hypothesized to form an aqueous channel, by assembling at least 5 bilayer spanning domains, probably of the same protein, but possibly even in an inter-protein manner, as the presence of dimers and tetramers has been described (Pessino et al., 1991; Herbert and Carruthers, 1991) in isolated cells.

It is localized in almost all the body tissues, even if, in any case, it is low-expressed and it is found in co-presence with a more highly tissue specific transporters (Muekler, 1994); it was found in all embryo tissues of mouse, from the oocyte onward. It has been also found in the basal membrane of hepatocytes surrounding the central venule, thus evidencing a peculiar role of this transporter in body barriers dividing blood flow from cell. In fact, another very important localization of GLUT 1 is the blood-brain barrier, where it supports the energy basal maintenance of brain cells (that are very sensible to glucose lacking). The regulation of GLUT 1 has been studied in culture cells treated with various enhancers or depressive molecules (Muekler, 1994), while in vivo studies have been performed in rat to look for the variations in mRNA expression (Birnbaum et al., 1986). It is stated that low levels of glucose stimulate this carrier expression, while high levels of the substrate do not (Muekler, 1994).

GLUT 2 is another member of the class 2 subfamily: this interesting transporter has a low affinity for glucose, can transport fructose (Wood and Trayhurn, 2003) and, with a higher affinity, glucosamine (Uldry et al., 2002); its main distribution is in β-pancreatic cells, in the small intestine epithelium (baso-lateral membrane), liver and kidneys (Thorens et al., 1988; Thorens, 1992; Fukumoto et al., 1988). In these tissues glucose uptake is not dependent on the presence of the different transporters, but on glucose concentration in the fluids they are in contact (Sheepers et al., 2004).

GLUT 2 is involved in control of gluconeogenetic/glucolytic machinery of the body: in fact, after a meal, blood glucose concentration increases and, as a response, pancreatic β cells uptake glucose by GLUT 2 activity; this glucose, phosphorylated by glucokinase into glucose 6 P, ends in a inhibition of the ATP sensible K^+ channel, leading to an increase in Ca^{++} intracellular concentration and to insulin secretion. In liver cells glucose is up taken by GLUT 2 and metabolized through the anabolic glicogenosyntetic pathway, under insulin stimulus (Sheepers et al., 2004; Fig. 11).

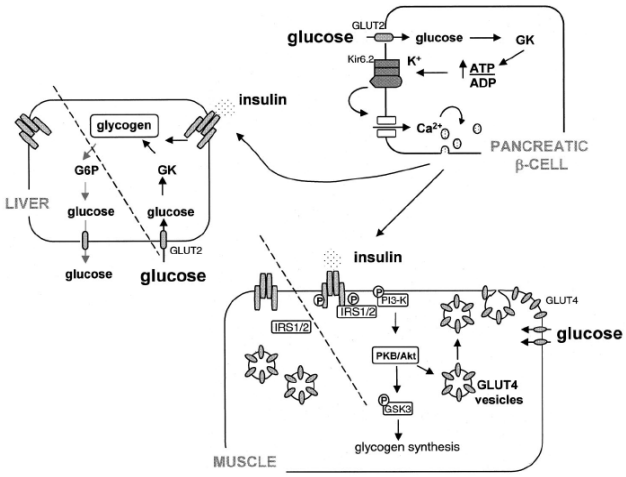

Fig. 11. Glucose sensitive machinery (*Scheepers et al., 2004*). Description of the different metabolic pathways of glucose utilization in insulin sensitive tissues.

This “sensitive” mechanism is therefore sustained by GLUT 2, low affinity – high capacity glucose transporter, and by glucokinase, whose capacity to convert glucose into glucose 6-P is lower than the transport capacity of the GLUT isoforms, and is consequently the real control point of the fine mechanism.

A similar mechanism is also present in brain: as brain cells are very sensible to glucose lack, there is a glucose-level controlling system that is supported by two kinds of neurons. The first one, glucose sensitive neurons, are activated by low glucose levels, while glucose responsive neurons are activated by high glucose levels; in this case, glucose responsive neurons behave as pancreatic β cells and induce the inactivation of the ATP sensible K^+ channel, with an increase of intracellular calcium and consequently an augmentation in neuronal firing. GLUT 2, as well as GLUT 3, seems to be involved in this very important regulation system (Sheepers et al., 2004).

In epithelial cells GLUT 2 is expressed only in basolateral membrane, that permits the passage of intracellular glucose to the blood circle; on the other side of the cell (Fig. 12), there is a SGLT transporter, where it acts by up-taking glucose from the intestinal lumen or from the ultra-filtrate (Thorens et al., 1990; Orci et al., 1990; Muekler, 1994; Diez-Sampedro, 2003; Sheepers, 2004).

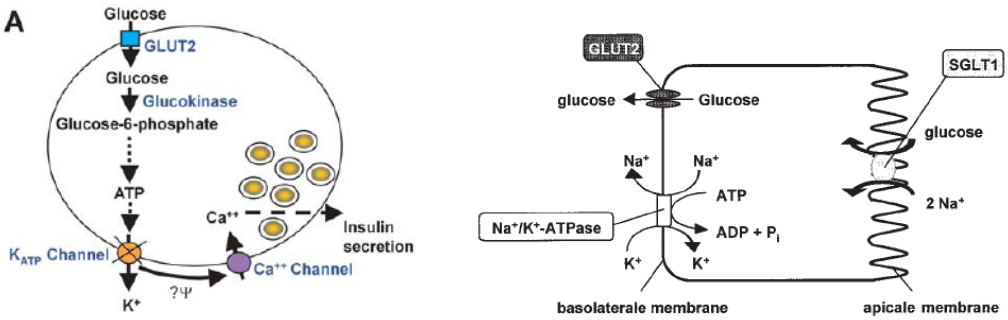

Fig. 12. GLUT 2 function (*Sheepers et al., 2004; Uldry and Thorens, 2004*). The figure underlines the important role of GLUT 2 in pancreatic β cells and intestinal epithelial cells.

Sheepers (2004) reports studies in which the importance of GLUT2 in diabetes is evident: knockout mice develop earlier diabetes and die within 3 weeks with lower levels of insulin and glucagon, as well as a fuzzy development of pancreatic islets.

GLUT 3 was first discovered by Thorens and coworkers (Kayano et al., 1988) and it was defined the neuronal glucose transporter. Because of its low k_m rate for glucose, it was stated that it is a high affinity glucose transporter, even if this definition is not completely correct (Muekler, 1994). GLUT 3 was also found in kidney, placenta and liver cells; a peculiarity of this transporter is that it is found in the cytoplasm of neurons, and it is exposed when these cells need it (Uldry and Thorens, 2004; Fig. 13); what's more, this protein is reported to transport mannose, galactose, maltose, xylose and dehydroascorbic acid.

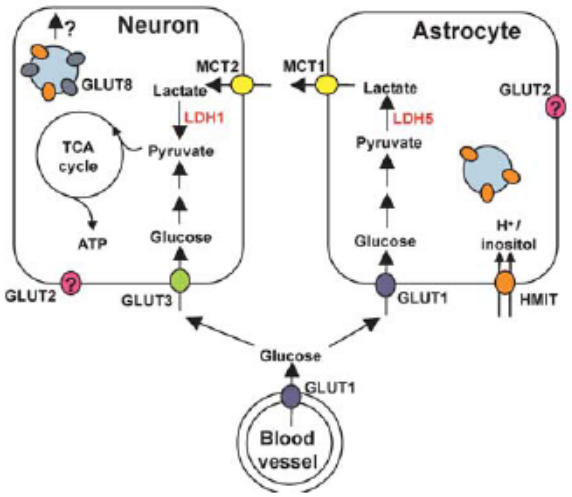

Fig. 13 GLUTs in neurons (*Uldry and Thorens, 2004*). The picture shows the important role of some GLUTs in supporting neuronal sugar supplying.

GLUT 4 is another member of class I subfamily and maybe it is the most interesting: in fact, it is a high affinity glucose transporter found in insulin-responsive tissues (Fukumoto et al., 1989) such as skeletal muscle, adipose tissue (brown and yellow) and heart muscle.
It is clearly much more expressed in fat than muscle, and in these insulin responsive tissues it is usually found with the most diffused transporter, GLUT 1 (Muekler, 1994). The characteristic of insulin sensitive tissues is that they can increase their glucose uptake in response to insulin stimulation (Uldry and Thorens, 2004). The base of this mechanism is GLUT 4 transposition from the inner cell structures, that is initiated by the activation of the insulin receptor consequently to the insulin-receptor binding (Fig. 14).

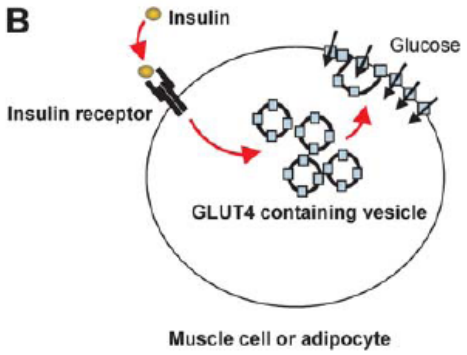

Fig. 14. GLUT 4 exposition in insulin sensible tissues (*Uldry and Thorens, 2004*).

As GLUT 4 vesicles are exposed to the cell surface, there is a 20 to 30 fold increase in glucose uptake in fat and muscle (Sheepers et al., 2004; Uldry and Thorens, 2004). If we take into account that muscle metabolizes from 20 to 90% of the whole body glucose in basal and hyper-insulinemic condition respectively, we can understand the importance of the mechanism of GLUT 4 translocation and function (Muekler, 1994). The actual mechanism of translocation of the GLUT 4 molecules is not yet fully clear: insulin-binding to its receptor stimulates an AMP dependent protein

kinase that induces the translocation of the tubule-vesicular cytoplasmic domains containing GLUT 4 molecules (Holman et al., 1990; Al-Hasani et al., 2002).

A lack of these isoforms is strictly related with type-2 diabetes (Sheepers et al., 2004; Uldry and Thorens, 2004), as there is a peripheral resistance to insulin, that ends in an iperinsulinemic-iperglycemic condition. This transporter has the ability to transport glucosamine and dehydroascorbic acid (Uldry and Thorens, 2004).

The last member of Class I proteins is GLUT 14: this transporter has been found only in man, and it is a mutation of the GLUT 3 gene. Its amino-acidic structure shares 95% sequence with GLUT 3, and its location is restricted to testis (Wu and Freeze, 2002; Sheepers et al., 2004)

Class II members are GLUT 5, that could be considered as the archetype of this subfamily, GLUT 7, GLUT 9 and GLUT 11.

GLUT 5 is an isoforms that has been discovered in human small intestine and demonstrated low glucose but good fructose transport activity (Kayano et al., 1990; Burant et al., 1992). It has been also found in insulin sensitive tissues and brain (Sheperd et al., 1992) but it doesn't seem to respond in an "acute manner" to insulin stimulus (Sheperd et al., 1992; Muekler, 1994). Its fructose specificity could be the explanation of its having the most different DNA sequence in the family (Kayano et al., 1990); moreover it represents the main machinery in fructose uptake in the small intestine, where it is usually localized in the apical membrane and works together with GLUT 2 (see Fig. 15).

What's more, GLUT 5 is not inhibited by cytochalasin B (as it happens for the class I transporters, Uldry and Thorens, 2004).

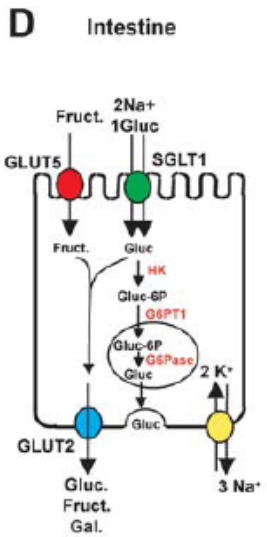

Fig. 15. Glucose/fructose transport in small intestine (*Sheepers et al. 2004*).

GLUT 7 is a high affinity glucose and fructose transporter, firstly identified and described by Cheesman group (Li et al., 2004). It is predominantly located in the small intestine epithelial cells, but it could also be found in gross intestine, testis and prostate (Sheepers et al. 2004).

GLUT 9 is a fructose transporter recently discovered (Phay et al. 2000; Carayannopoulos et al. 2004) to be particularly present in liver and kidney, even if low levels of its mRNA have been also found in placenta, small intestine, lung and leukocytes. It has been suggested (Carayannopoulos et al., 2004) that it plays a very important role in preiplantation, and some studies in mouse demonstrated that, of the 3 isoforms known so far, 2 demonstrated a glucose transport activity when expressed in oocytes (Carayannopoulos et al., 2004).

GLUT 11 is the last discovered member of class II transporters; it is expressed predominantly in heart and skeletal muscle (Doege et al., 2001), even if it could also be found in lung, brain, small intestine and peripheral leukocytes (Uldry and Thorens, 2004). It is present in 3 different isoforms, that demonstrate a different tissue-specificity (Uldry and Thorens, 2004); furthermore, as reported by Gaster et al. (2004), GLUT 11 shows a peculiar distribution among the skeletal muscle fibres. In fact, differently from GLUT 4, it is located in an intracellular domain and, to a lesser degree, in the plasmatic membrane (sarcolemma). Another interesting feature of this transporter is that it could be partially digested by a specific protein present in skeletal muscle (PNGase F), so that this could represent another mechanism of control of glucose metabolism (Wu et al., 2002).

Due to its low inhibition response to citochalasin D and to the inhibition of glucose transport by fructose, it has been stated that this transporter is a low affinity glucose – high affinity fructose transporter (Doege et al., 2001).

As reported by Joost and Thorens (2002), Class III transporters are the most different of the family, on the basis of genetic sequence and structural features: in fact, they have a very short extracellular domain in loop 1, lacking in a glicosilation motif, that is transposed in loop 9. This class is retained to be the most ancient in the phylogenesys of the GLUT family, as its members share a lot of characteristics with bacteria, yeasts and Drosophila proteins and their adaptation to mammalian request

for glucose could be represented by the class I and II transporters (Joost and Thorens, 2002).

GLUT 6 was firstly and erroneously designed as GLUT 9 (Doege et al., 2000); this transporter has a low glucose affinity and it is not inhibited by cytochalasin B (Uldry and Thorens, 2004). It has been stated that, as for GLUT 8, it is retained in intracellular structures in adipocytes under experimental conditions, and that the necessary stimulus for its exposure has not been well understood yet (Lisinski et al., 2001). It is distributed in brain, spleen and peripheral leukocytes (Uldry and Thorens, 2004).

GLUT 8 is a high affinity glucose transporter, whose activity can be interfered by mannose and fructose, thus indicating its poli-hexose transport activity (Doege et al., 2000b). It is mainly expressed in testis even if its presence is also reported in skeletal and heart muscle and in pre-implantation embryos (Carayannopoulos et al, 2000; Sheepers et al., 2004). It was also found in mature mouse spermatozoa (Shürmann et al., 2002).

GLUT 10 is prominently found in liver and pancreas and its gene has been related to type II diabetes (Dawson et al., 2001); it was also found in embryonic tissues (brain and liver) as well as in many other tissues as skeletal muscle, brain and heart (Uldry and Thorens, 2004).

GLUT 12 expression is related to heart and prostate and in experimental conditions it has been found to transport glucose and to be inhibited by mannose and fructose (Rogers et al., 2002; Sheepers et al., 2004). GLUT 12 has been also demonstrated in prostate carcinoma (Chandler et al., 2003) and breast cancer (Rogers et al., 2003).

The last transporter related to GLUT family is HMIT, whose gene name is SCL213. This transporter is a H^+-myoinositol syntransporter, especially expressed in brain and seems to lack any sugar transport activity (Uldry et al, 2001).

GLUT in sperm cells

As already seen when talking about sperm metabolism, sperm cells use hexoses as an energy source, which is mainly dedicated to movement and to achieve fertilizing ability.

Spermatozoa utilize external sources of hexoses, because during their maturation they lost the majority of their cytoplasm without accumulating energy sources.

The first study on energy uptake by sperm cell was carried out by Petersen and Freund (1975), who understood that spermatozoa uptake energy substrate from the environment in which they are. In late 70s and 80s some studies have been performed to clarify both presence and function of sugar transporters in sperm cells or in gamete progenitors (Petersen et al., 1977; Nakamura et al., 1987), and they especially focused on function and inhibition of sugar transport. These studies were very important in assessing the capability of sugar uptake by sperm cells as well as to understand the possible selectivity of sugar transport and utilization in metabolic strategies/pathways.

The first report on the presence of a sugar transporter belonging to GLUT family is the work by Burant and co-worker (1992); they found that GLUT 5 is present in human testis, as well as in ejaculated spermatozoa. They demonstrated this with western blotting studies, in which they found a reactive band of about 50 kDa; they didn't give information on the subcellular localization in sperm cells, but only in testis sections, where it corresponds with the site where late spermatids are.

GLUT 3 was also demonstrated to be present in human and rat sperm cells (Haber et al., 1993), as well as in testis (MW of about 48 kDa).

The first wide study on GLUT in mammals mature spermatozoa is Angulo's and co-workers' one (1998). In that paper man, rat and bull sperm cells were studied, as well as testicular tissues, in order to better understand the location of these proteins among the cell membrane, to characterize their presence with a comparative study and to assess they functional capacity to transport sugars and vitamins (Glander and Detmer, 1978; 1978a; Vera et al., 1993).

Those Authors studied 5 GLUTs, the class I component GLUTs 1, 2, 3 and 4 and GLUT 5, the fructose transporter. They studied mRNA expression in testis, its distribution in testicular tissue and presence and distribution in spermatozoa.

As for human sperm cells, they found GLUT 1 in acrosomal region and principal and endpiece of the tail; GLUT 2 showed a similar distribution pattern, while GLUT 3 was found in the midpiece. GLUT 4 didn't show any immunological positivity, while GLUT 5 was detected in subequatorial region, mid and principal piece. These results were also supported by western blot analysis. Sperm membrane proteins were tested with the same antibodies used in immunocytochemistry, giving the following results: two bands of 52 and 60 kDa, respectively, were associated with GLUT 1; a single band of almost 60 kDa was detected for GLUT 2; two bands of 54 and 62 kDa for GLUT 3 and a band of about 60 kDa for GLUT 5. No reactive specific bands were detected for GLUT 4.

As for rat sperm cells, the Authors only performed immunocytochemistry on sperm cell, obtaining the following results: GLUT 1 was present in the apical region of the head and in the principal and end piece of the tail; GLUT 2 was detected in the acrosomal region, while GLUT 3 in the midpiece and, with a fainter signal, in the principal piece. No signal was detected for GLUT 4 , while GLUT 5 was found in the sperm head and in the midpiece.

Studies on bull spermatozoa evidenced that GLUT 1 was present, as in the other species, in acrosomal region and principal and endpiece of the tail; GLUT 2 showed a weak positivity in the head and midpiece, while GLUT 3 presented a very strong positivity in the same loci; even in bull GLUT 4 did not express any positive signal, while GLUT 5 resulted to be present in sperm head and midpiece, with a weaker signal in the principal piece.

This study, being the first in describing GLUT distribution among sperm cells membrane, is mainly focused on sugar uptake by spermatozoa and on the inhibition of this mechanism by a series of molecules. This approach directly links GLUTs presence and sperm cells metabolic activity, which was later deepened in other works.

In 2002, Rigau and co-worker, studied dog sperm metabolism with particular attention on glucides metabolism and motility.

They found GLUTs 3 and 5 to be present in dog sperm cells' membrane, as well as the sodium-glucose transporter SGLT-1. GLUT 3 showed a reactive band at 45 kDa, while GLUT 5 showed bands of 35 and 45 kDa; finally, SGLT-1 was detected in two bands of 50-60 kDa. The localization of these transporters among the cell membrane, as already seen in human, rat and bull spermatozoa, is peculiar for each

isoform: GLUT 3 showed a strong positivity in the midpiece and principal piece, while GLUT 5 was present in the peri-acrosomal region and midpiece. SGLT-1 signal was found in the peri-acrosomal region, equatorial line and midpiece.

These findings led those Authors to support the idea that different metabolic ways are present in dog spermatozoa and that each way has a peculiar and precise substrate. The location of GLUT 3 high affinity glucose transporter is quite different from that of GLUT 5, that is a specific fructose transporter, as the first one is not present in sperm head . What's more, dog spermatozoa undergo a strong motility activation if incubated with fructose (Rigau et al., 2001), while the activation under the effect of glucose is less intense. On the other side, protein phosphorylation is higher with glucose than fructose, and the rate of sugar phosphorylation is more efficient (Rigau et al., 2002). These differences may lead to the knowledge of the fine regulatory mechanisms lying under sperm function and metabolic changes. Those Authors believe that the different way used to uptake sugars could be the regulatory machinery in hexoses metabolism. In that work, the authors didn't find any positive signal against GLUT 1, 2 and 4 in dog sperm cells.

Another work of the same group (Medrano et al., 2006) has studied the presence and distribution of glucose transporter GLUT 3 in boar spermatozoa. In that study the presence of the transporter was demonstrated by western blot analysis (a positive band at about 45 kDa) and immunocytochemistry, that showed a distribution pattern mainly expressed in sperm head and midpiece. What's more, with the immunogold technique, they showed the internal acrosomal membrane and cytoplasmic membrane localization of the protein, as well as its intracellular localization in the midpiece, just through cell and mitocondrial membranes. GLUT 3 was also found in non soluble fractions, that are non-membrane structure, thus supporting the hypothesis that GLUT 3 could behave as GLUT 4 and being exposed to the cell surface as it is necessary. Even if this "behavior" has not been described in GLUT 3 since now and the activating stimulus is not known, the Authors believe that this difference marked in membrane and metabolism attitudes in sperm cells may explain such a new and strange finding.

As already seen in the "metabolism" chapter, the main control point in sperm metabolism is represented by enzymes involved in the glycolitic process, such as hexokinase, pyruvate kinase and lactic dehydrogenase. The most important enzyme in boar sperm cells is hexokinase I that, with its high affinity for the substrate,

characterizes boar sperm metabolism as pre-eminently glycolitic (Fernandez-Novell, 2004). In this regard, it is stated that GLUTs can represent a previous control point in sperm metabolism, as they modulate substrate availability and uptake (Medrano et al., 2006).

In a recent study on the effects of cryopreservation in boar sperm cells, Sancho and co-workers (2007) analyzed GLUT 3 and 5 expression and location pre- and post-thawing. It is well known that thawing induces membrane changes in sperm cells, that are particularly evident in lipid distribution and protein re-location (Parks and Graham, 1992; Watson, 1995). In their work, Sancho et al. (2007) found GLUT 3 to be present in the acrosomal membrane and principal and endpiece of the tail with a strong immunolabelling, while in the post-acrosomal region, connecting and midpiece, the immunoreactivity was lower in fresh ejaculated boar sperm. The same location was observed during refrigeration at 5 °C, while a strong change was reported in GLUT 3 location and signal strength after thawing: in fact, the cryoconservation process induces a redistribution of the signal among sperm membrane and a loss of intensity.

As for GLUT 5, it was found in the acrosomal membrane, equatorial band and in the whole tail, and its localization after refrigeration and thawing processes didn't seem to change (Sancho et al., 2007).

The Authors explained these findings as a consequence of the different structures in which the two different GLUTs are involved: GLUT 5 may be anchored to cytoskeletal structures, that don't permit its movements, while GLUT 3, being a more membrane-associated protein, could be relocated or lost during the thawing process. These last findings are corroborated by the western blot analysis, in which the positive GLUT 3 signal markedly decreases after thawing, while GLUT 5 one does not.

Another interesting study took into account both activity and location of a novel member of the GLUTs family, GLUT 8 (Schürmann et al., 2002). Those Authors discovered the protein in human tissues in 2000 and then studied its expression in various tissues, reaching the conclusion that GLUT 8 is mainly expressed in testis and, to a lower extent, in liver, muscle, heart and adipose tissues. They demonstrated by northern blot analysis that GLUT 8 mRNA is expressed in testis, but in particular it appears in mature cell lines (spermatids and spermatozoa) and that the expression of the protein is strictly related to the action of gonadotropins (Schürmann et al., 2002).

The immunocytochemical trials showed that GLUT 8 is present in man and mouse mature spermatozoa, with an intense signal in the acrosomal membrane and a fainter signal in the post acrosomal region and tail.

GLUT 8 is predominantly a glucose transporter, with a high affinity, that can be inhibited by fructose, thus suggesting that it transports both sugars; in addition, it was found that the transporter is usually located in intracellular compartments (Lisinki et al., 2001), which led the Authors to hypothesize that the protein could have a similar activity control even in sperm cells.

Finally, in their paper those Authors introduced a concept that has been later deepened: that spermatozoa have a compartmentalization of the glycolitic activity, that could be explained by the different localization of fructose and glucose transporters, that could find themselves strategically located nearby the metabolic inner pathway.

No other studies regarding GLUTs and spermatozoa are present in literature till now, even if the mechanism of their function in sperm cells is not fully clarified and there is a lack of knowledge concerning the isoforms present in male gamete.

Aims of the study

This study was aimed at a better understanding of mammal sperm cells physiology, by both deepening the knowledge in those species in which studies on GLUTs have already been performed and furnishing new information on some other species in which these arguments have not been approached yet.

To study in a more complete manner sperm physiology, in vitro capacitation and induction of acrosome reaction were performed: as already seen, the changes in sperm cell metabolism are evident during sperm capacitation and acrosome reaction, even if they are not yet fully characterized from a metabolic point of view. This is why we chose to study possible changes during capacitation and acrosome reaction.

Before performing our experiments, an overall sperm study including viability assessment, acrosome integrity, total motility and (only for donkey sperm) some CASA (Computer-Assisted Sperm Analyzer) parameters evaluation, was performed to assess the ejaculate quality and to verify in the most conservative way the physiological state of the spermatozoa.

Summarizing, the aims of this work were to:

- Study the presence of GLUTs 1, 2, 3, 4 and 5 in boar, dog, stallion and donkey sperm cells as well as their distribution on the cell membrane in freshly ejaculated spermatozoa;
- Evaluate possible changes in GLUTs 1-5 distribution after capacitation and acrosome reaction in boar, stallion and dog sperm cells;
- Evaluate the possible effects of insulin and IGF on GLUTs localization in boar spermatozoa;
- Assess possible change in GLUTs localization due to sex sorting process in boar spermatozoa.

Materials and methods

All the reagents were obtained from Sigma Chemical Co (St. Louis, MO, USA), unless otherwise specified.

Semen collection and preparation

Boar

Sperm rich fraction of at least 7 ejaculates was obtained weekly with the gloved-hand technique from mature boars (aging from 9 months to 3 years). Semen was brought into the lab within 15 min and diluted 1:1 in Androhep^{TM} (Minitüb, Tiefenbach, Germany).

Stallion

Horse semen was collected using an artificial vagina, from horses of an average age of 8 years with a weekly frequency and filtered right after the collection. Semen was delivered within 30 min to the lab and diluted 1:1 in Tyrode medium (Rathi et al., 2001).

Dog

Dog semen was collected from voluntary donors in the Didactic Hospital of the Faculty of Veterinary Medicine at Bologna University, so that it couldn't be collected at precise intervals of time. It was collected manually and brought to the laboratory within 15 min and diluted 1:1 in Tris Glucose medium.

Donkey

Martina Franca donkey semen samples were obtained from 6 adult and fertile jack assess (4-8 years of age, 380-450 kg in weigh) using a Missouri artificial vagina in presence of a jenny in natural estrus. After collection semen was filtered through a sterile gauze and gel-free volume was recorded. Subsequently spermatozoa were diluted 1:10 with INRA 96 medium (INRA – IMV Technologies, L'Aigle, France).

After that, samples were divided into aliquots to perform immunocytochemistry and western blotting.
Boar, stallion and dog samples were also aliquoted to induce capacitation and acrosomal reaction.

Sperm evaluation

Sperm concentration
Sperm concentration was determined by a haemocytometer (Burker chamber – Merck, Leuven, Belgium).

Sperm plasma membrane integrity
Sperm viability was evaluated by incubating 25 µL of semen with 2 µL of a 300 µM propidium iodide (PI) stock solution and 2 µL of a 10 µM SYBR GREEN-14 stock solution, both obtained from the live/dead sperm viability kit (Molecular Probes, Inc, Eugene, OR, USA), for 5 min at 37°C in the dark. After incubating for 3-5 min, 10 µL of sperm suspension, after mounting, were analysed with a Nikon epifluorescence microscope. The spermatozoa with green or red fluorescence on the head were considered live or dead, respectively.
As for donkey spermatozoa, they were subsequently fixed with 1 µl of 3% glutaraldehyde and a 6 µl drop of stained semen was placed on an slide, covered with a coverslip and examined under an Olympus BX51 epifluorescence microscope. At least 200 cells were counted: spermatozoa with bright green fluorescence (SYBR – 14) were considered viable, those partially or totally red (PI) were considered dead.

Donkey sperm motility assessment
Sperm motility was evaluated using a CASA system IVOS 12.3 (Hamilton-Thorne Bioscience, Beverly, MA, USA), set at 60 frames per sec (Hz) and 30 frames per field. 2 µl of diluted semen were loaded in a 4-chamber 20 µl slide (Leja, Nieuw-Vennep, The Netherlands) and 12 non-consecutive fields were analyzed. Progressive motility (PMOT, %), average path velocity (VAP, µm/s), straightness (STR, %) and rapid cells (RAPID, %) were recorded. Spermatozoa with VAP > 75 µm/s and STR > 80% were considered progressive.

Donkey sperm morphology

For morphology evaluation, an aliquot of semen was fixed in buffered-formol saline solution (Hancock, 1957) and examined under phase-contrast microscope at magnification of 1000x. The morphological abnormalities were counted as a percentage on at least 200 spermatozoa (Malmgren, 1997).

Induction of in vitro capacitation and acrosome reaction (AR) in boar, stallion and dog semen

Each boar and stallion sample was divided into 4 aliquots, 2 of which were used to obtain capacitated samples, and 2 for acrosome reacted samples. Then, one aliquot of capacitated semen and one of AR were used to perform western blot analysis and the remaining were used for immunocytochemistry. As for dog, only immunocytochemistry was performed.

Boar

Boar diluted semen was washed twice at 800 x g for 3 min in capacitating medium (Brackett and Oliphant's medium supplemented with 12% foetal calf serum (Invitrogen srl, San Giuliano Milanese, MI, Italy) and 0.7 mg/mL caffeine). The pellet was then resuspended in 2 mL of Brackett Oliphant's medium (BO) at a final concentration of 100 x 10^6 spermatozoa/mL and incubated at 39°C in a humidified atmosphere of 5% CO_2 for 3 h in order to induce in vitro capacitation.

Two aliquots were added with 10 µM calcium ionophore A23187 for the last 30 min of incubation to induce AR.

Stallion

A modified Tyrode medium added with bicarbonate (Tyr + bic) was used for inducing capacitation in stallion spermatozoa (Christensen et al., 1996). Two mL of stallion sperm were transferred into a 15 mL tube, mixed with 6 mL of Tyr (without bicarbonate) and centrifuged at 900 x g for 10 min to allow the removal of seminal plasma and washed twice. The final pellet was resuspended in Tyr + bic to a final concentration of 100 x 10^6 spermatozoa/mL and then incubated at 37°C for 4 h.

After 3,5 h two aliquots were incubated for 30 min with 10 µM calcium ionophore A23187 to induce AR.

Dog

Canine spermatozoa were suspended in Tris-glucose and washed twice by two successive centrifugations at 200 x g for 10 min. The resulting pellet was resuspended in the capacitating medium (1-CCM) at a final concentration of 60-80 x 10^6 spermatozoa/mL and incubated for 4 h at 38.5°C in a 5% CO_2 atmosphere (Rota et al., 1999).

After 3h and 45 min, 10 µM calcium ionophore A23187 were added for 10 min to obtain AR cells.

IGF and Insulin-stimulated capacitation in boar semen

Boar samples were divided into 3 aliquots, each containing 100 x 10^6 spz/ml. The first aliquot was fixed and represented the fresh semen control; the second one was capacitated under the same condition above described; the third aliquot was added with 100 ng/ml IGF or 10 µg/ml insulin. After incubation, aliquots were fixed for immunofluorescence.

Capacitation assessment

The degree of capacitation was assessed by different methods depending on the species: boar spermatozoa on the basis of Hsp-70 immunolocalization (Spinaci et al., 2005), stallion and dog sperm by chlortetracycline (CTC) staining.

Indirect immunofluorescence was performed in boar spermatozoa as previously described (Spinaci et al., 2005). During capacitation and, further on, acrosome reaction, Hsp70 undergoes a redistribution with typical patterns that have been demonstrated to be strictly related to the functional status of sperm. Hsp70 immunoreactivity in uncapacitated spermatozoa is confined to a well-defined triangular-shaped area in the equatorial segment (uncapacitated pattern) while capacitated spermatozoa exhibit the reactivity in the equatorial line sometimes associated with a fainter triangular signal and/or a semicircular line on the anterior boundary of the equatorial segment (capacitated pattern).

For dog and stallion spermatozoa, CTC method was carried out as follows. Briefly, 50 µL of semen suspension was mixed with the same amount of CTC solution (750

µM CTC in a buffer of 20 mM Tris-HCl, 130 nM NaCl, 5 mM L-cysteine); after 30 sec, 10 µL glutaraldehyde were added and then 10 µL of semen were placed onto a slide in a drop of Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). After mounting, the slides were analysed using an UV set filter. Spermatozoa were considered as capacitated not only when fluorescence appeared on both acrosome and tail, but with a fluorescence free band on the post acrosomal region.

Acrosome reaction

The occurrence of AR was confirmed with fluorescein isothiocyanate (FITC)-conjugated agglutinin derived from Pisum sativum (FITC-PSA) staining. Briefly, spermatozoa were fixed for at least 30 min at -20°C in 95% ethanol and dried onto a microscope slide, then incubated with FITC-PSA solution (1 mg PSA- FITC/10 mL H_2O) for 15 min under dark conditions at room temperature. The slides were observed with the above described epifluorescence microscope. Fixed spermatozoa with intact acrosome were considered non-reacted, while those with total or partial loss of acrosomal fluorescence were considered as reacted (Cross and Watson, 1994).

Immunocytochemistry

All the procedures were carried out at room temperature unless otherwise specified. Sperm cells were spotted onto a poly-L-lysine-coated slide, fixed with absolute methanol at -20°C for 5 min and then with acetone for 30 sec. The slides were washed with PBS, let dry and then blocked with 10% (v/v) foetal calf serum (FCS) in PBS for 30 min.

Rabbit anti-human GLUTs 1, 3, 4 and 5 polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and goat anti-human GLUTs 2 and 4 polyclonal antibody (Santa Cruz Biotechnology) were added at the proper dilution in PBS 10% FCS. The incubation was carried out overnight at 4°C in humid chambers. After extensive washing, sperm cells were incubated with a goat anti-rabbit (GLUTs 1, 3, 4 and 5; dilution 1:2,200) and a rabbit anti goat (GLUTs 2 and 4, dilution 1:800) FITC-conjugated secondary antibodies for 1 h under dark conditions at room

temperature. Slides were then washed extensively with PBS and mounted with Vectashield mounting medium with propidium iodide. Control slides were treated similarly with the omission of the primary antiserum. Images were obtained using a Nikon digital camera installed on a Nikon epifluorescence microscope.

Western blot analysis

Each boar, stallion and donkey semen aliquot was sonicated (30 pulses with 70% power) in 200 µL homogenization buffer (1 mM EDTA, 1 mM EGTA, 50 mM Tris, 5 mM PMSF) and then centrifuged at 13,000 x g for 15 min at 4° C. The supernatant was separated from the pellet that was resuspended in 200 µL homogenization buffer. Each fraction was added with SIGMA anti-protease cocktail (40 µL) and frozen at -80 °C until use.

Protein concentration in supernatants and pellets was measured according to Lowry et al. (1951). Bovine serum albumin (BSA) was used as a standard. Each sample was tested at 750 nm in triplicate using 96-well microtiter plates in a MultisKan EX spectrophotometer.

Appropriate volumes of supernatants and pellet extracts were loaded in each well to obtain 30 µg protein/lane.

SDS-PAGE was performed using 10% Bis-Tris gels with MOPS as running buffer, under reducing conditions at 200 V for 50 min. After electrophoresis, proteins were transferred to nitrocellulose membranes 30 V for 1 h in an Invitrogen Xcell SureLock Blot Module using transfer buffer, pH 7.2. Blots were briefly washed in PBS and the non-specific protein binding was blocked with 4.5% milk powder in PBS-T20 (PBS 0.1% Tween 20) for 3 h at room temperature.

Membranes were then incubated with polyclonal rabbit anti-human GLUTs 1, 3, 4 and 5 antibodies (Santa Cruz Biotechnology) and goat anti-human GLUTs 2 and 4 antibody (Santa Cruz) diluted 1:500 in Tris-buffered saline T-20 (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.1% Tween 20) overnight at 4°C. After several washings with PBS-T20 the membranes were incubated at room temperature with 1:20,000 biotin-conjugated anti-rabbit secondary antibody (Stressgen Bioreagents, Ann Arbor, MI, USA) for GLUTs 1, 3, 4 and 5 and 1:10,000 anti goat biotin-conjugated secondary antibody (BioFix Laboratories, Owing Mills, MD, USA) for GLUTs 2 and 4. After several washings the membranes were incubated with 1:1,000 diluted anti-biotin

streptavidin horseradish peroxidase (HRP)- conjugated (Cell Signalling Technology, Danvers, MA, USA).
The western blots were developed using Super Signal West Pico (Pierce Biotechnology, Rockford, IL, USA) according to manufacturer's instructions.

Flow sorting

A MoFlo SX flow cytometer/sperm sorter (DakoCytomation Inc, Fort Collins, CO, USA) equipped with an argon laser (wavelength 351 at 150 mW) and modified especially for sorting sperm (Johnson and Pinkel, 1986; Johnson and Welch, 1999) was used. Dulbecco phosphate buffered saline (DPBS) served as sheath fluid. The instrument sheath pressure was 40 psi, and the trigger rate was adjusted to 20,000 cells per second. Sorted spermatozoa were deflected into 20-mL polypropylene tubes containing 500 µL of 2% Tes-Tris-egg yolk buffer (Johnson, 1991) supplemented with 10 µL of frozen-thawed boar seminal plasma. After collection of 7–8 x 10^6 sperm per tube, the 2 populations were pooled (since sex predetermination was not an objective of the experiment). The samples were centrifuged at 800 x g for 10 min, and the pellet was resuspended with Androhep. Spermatozoa were counted and were fixed as below described for immunofluorescence staining. Control semen was kept at 16°C until processed.

Experimental design

Experiment 1: Detection of GLUTs 1, 2, 3, 4 and 5 by western blotting and their immunolocalization in boar, dog, stallion and donkey sperm cells

Experiment 2: Evaluation of GLUT re-localization after capacitation and acrosome reaction in boar, stallion and dog spermatozoa

Experiment 3: Determination of the effects of sex sorting procedure and stimulation with either IGF or insulin on GLUT localization in boar spermatozoa

Experiment 1

Detection of GLUTs 1, 2, 3, 4 and 5 by western blotting and their immunolocalization in boar, dog, stallion and donkey sperm cells

Results

Viability was assessed before performing any trial, as a pre-screening test.
Freshly ejaculated sperm cells viability, assessed by SYBR-GREEN14-PI method, was 86.4±1.8 %, 83.8±2.3 % and 80.6±2,9 % for boar, stallion and dog, respectively.
Martina Franca donkey semen characteristics are expressed in table 1. CASA analysis were performed in University of Teramo laboratories.

Semen parameter	**Mean±S.D.**
Volume gel free (ml)	96.7±23.1
Concentration (x10^6 spermatozoa/ml)	346±152
PMOT %	73.7±6.3
VAP µm/s	136±7
STR %	83.7±3.8
Rapid cells %	93±2.6
Viability (%)	88±3.8
Normal cells %	84.5±5
Abnormalities (total) %	8.5±1.2
Cytoplasmic droplets %	7±5.7

Western blotting

Our investigations demonstrated the presence of immunoreactive bands in non-sonicated pellets and in sonicated supernatants; no bands in non-sonicated supernatant and only very weak bands in sonicated pellet were observed, thus confirming the localization of GLUTs in sperm membrane.

Bands of the expected molecular weight for GLUTs 1 and 2 in boar, stallion and donkey spermatozoa were evident. In particular, GLUT 1 showed a reactive band around 50 kDa, GLUT 2 at about 45 kDa and a second reactive band at about 90 kDa, which can represent a dimer of this transporter; GLUT 3 was reactive in a band of about 45 kDa in pig, as well as in horse, while in donkey the specific reactive band was found at 65 kDa.

GLUT 5 showed a positive band of more than 60 kDa, both in stallion and boar sperm, while in donkey the specific band is at 65 kDa.

GLUT 4 didn't show any specific reactivity in the tested samples, with none of two antibodies that were tested. Only a very weak and aspecific band of 70/80 kDa was detectable in horse.

Negative controls were performed by omitting the primary antibody (data not shown) to check the absence of a secondary antibody aspecific signal.

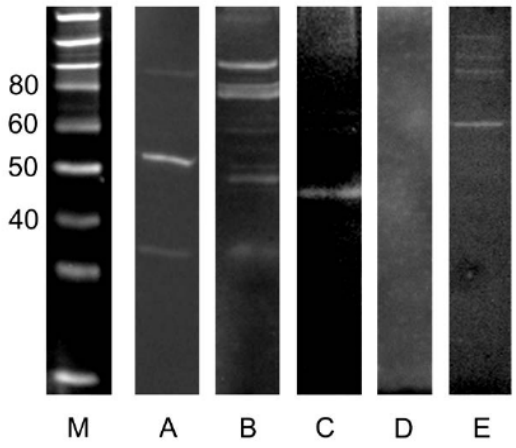

Figure 16

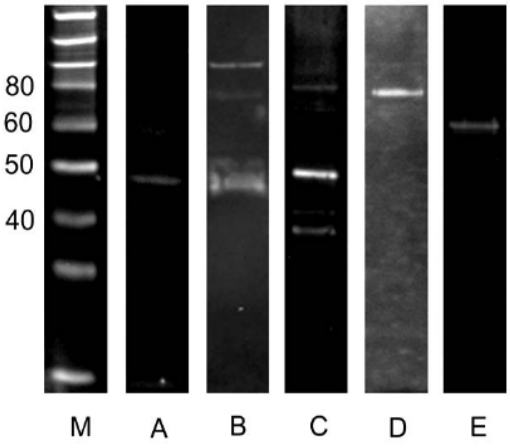

Figure 17

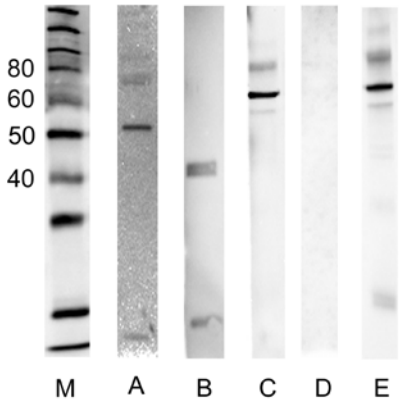

Figure 18

Figure 16. Representative western blotting of GLUTs 1 (A), 2 (B), 3 (C), 4 (D) and 5 (E) in boar spermatozoa. M molecular weight markers.
Figure 17. Representative western blotting of GLUTs 1 (A), 2 (B), 3 (C), 4 (D) and 5 (E) in horse spermatozoa. M molecular weight markers.
Figure 18. Representative western blotting of GLUTs 1 (A), 2 (B), 3 (C), 4 (D) and 5 (E) in donkey spermatozoa. M molecular weight markers.

Immunolocalization of GLUT 1

The localization of this transporter was similar in boar and stallion spermatozoa. In both species the signal was localized along the whole sperm tail and, with a spotted pattern, in the acrosomal membrane. In all stallion spermatozoa, a strong spotted positivity in the neck of the tail was observed. In canine spermatozoa, a very strong signal was evident at the apical ridge of the acrosomal membrane, while the rest of was negative; the tail showed a very faint signal in all its parts. In donkey a strong positivity signal was detectable in the apical part of the acrosomal membrane (AM) and a fainter one in the principal and end piece of the tail, while the midpiece resulted less positive.

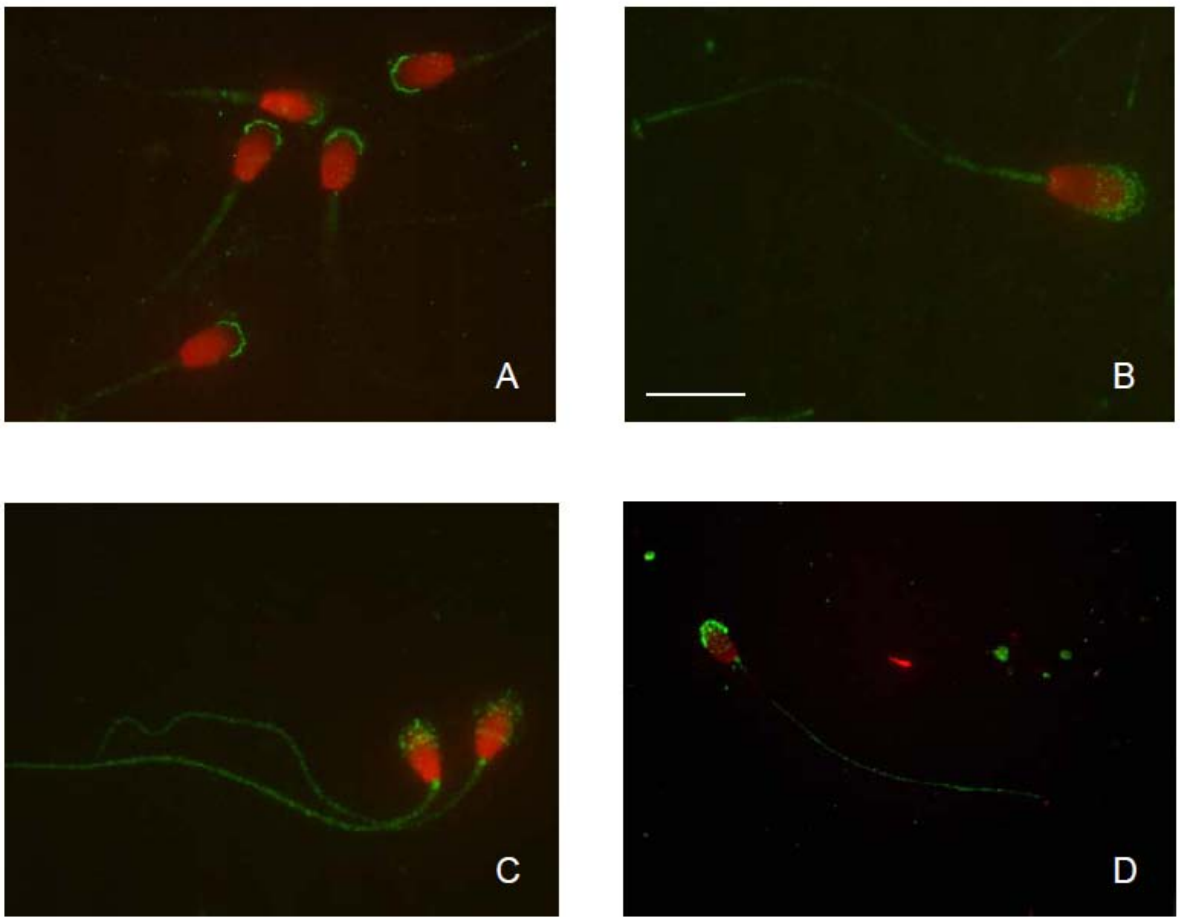

Figure 19. Representative photographs of GLUT 1 distribution in dog (A), boar (B), stallion (C) and donkey (D) spermatozoa. (Bar 10 μm)

Immunolocalization of GLUT 2

The localization of this low-affinity glucose/fructose transporter was similar in dog and stallion, but not in boar spermatozoa. In fact, an evident positivity in the acrosomal membrane, similar but stronger than that of GLUT 1, was observed in boar sperm. In stallion and dog the positive signal was present in the acrosome and in the tail; in this last species, a positive spot, localized at the beginning of the tail, was also shown.
In donkey sperm cells GLUT 2 showed a positivity in the AM and the signal was detected in the tail and in the equatorial line.

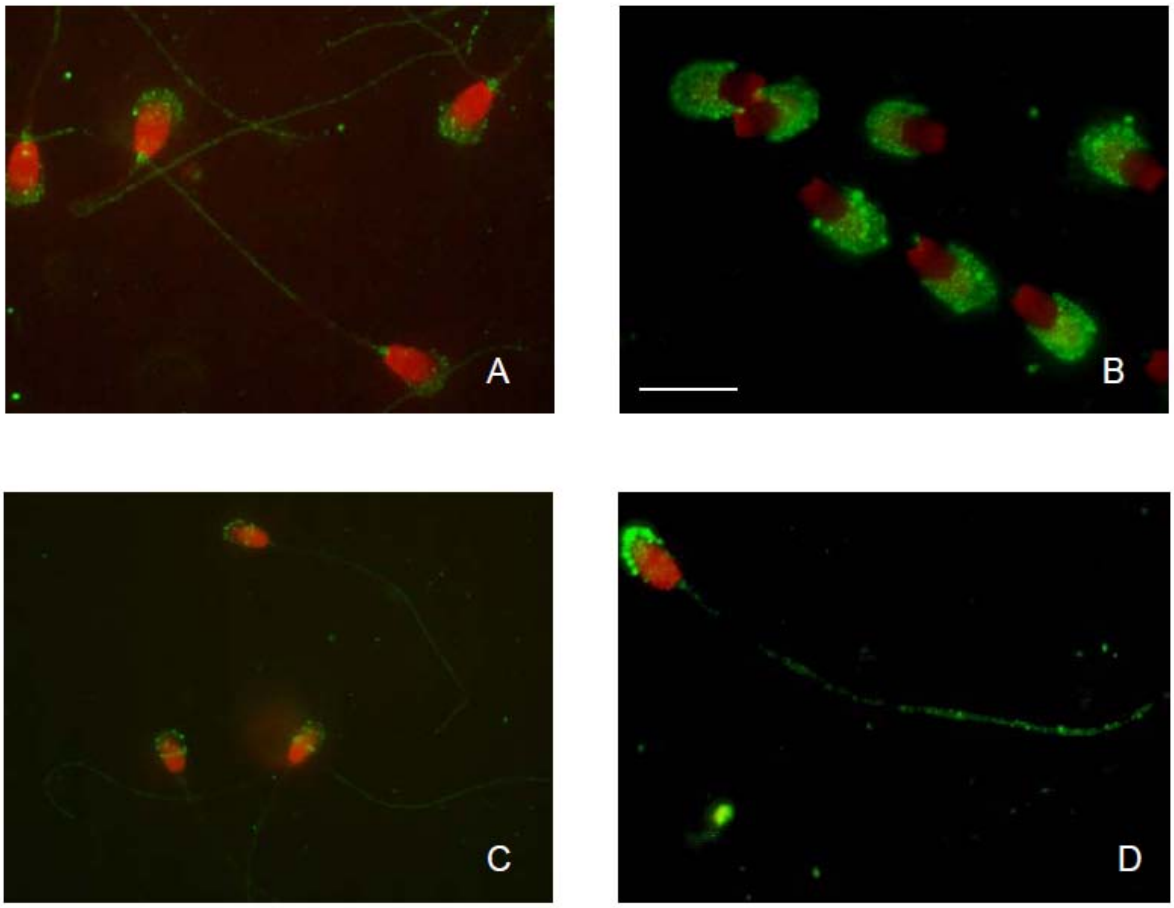

Figure 20. Representative photographs of GLUT 2 distribution in dog (A), boar (B), stallion (C) and donkey (D) spermatozoa. (Bar 10 μm)

Immunolocalization of GLUT 3

The localization was different in the four species examined. In boar spermatozoa the positivity was evident in the acrosome and in a subequatorial band of the sperm head, while a faint immunoreactivity was present in the tail. In stallion sperm cells a strong signal was evident in the tail, with a particular highlighted neck spot, while acrosome and equatorial band immunoreactivity was fainter. In dog spermatozoa the positive signal was present only in the principal and end-piece of the tail, with the exclusion of the mid-piece. As for donkey, GLUT 3 was localized in the apical external part of AM, in the tail, with a fainter midpiece, and in a very evident equatorial band.

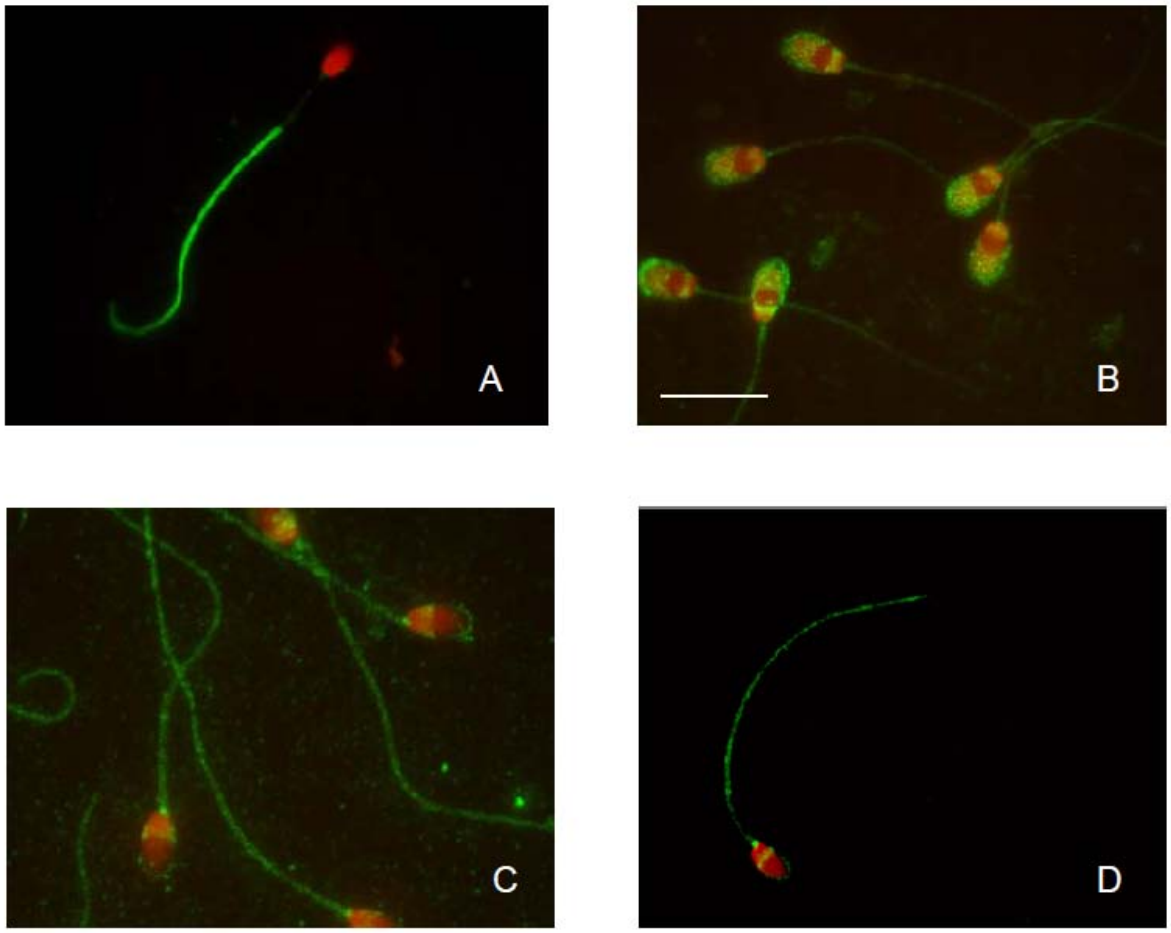

Figure 21. Representative photographs of GLUT 3 distribution in dog (A), boar (B), stallion (C) and donkey (D) spermatozoa. (Bar 10 μm)

Immunolocalization of GLUT 4

Weak immunoreaction in horse and donkey spermatozoa, while a faint signal was present in the mid-piece and in the acrosomal membrane in boar and dog spermatozoa due to aspecific reaction

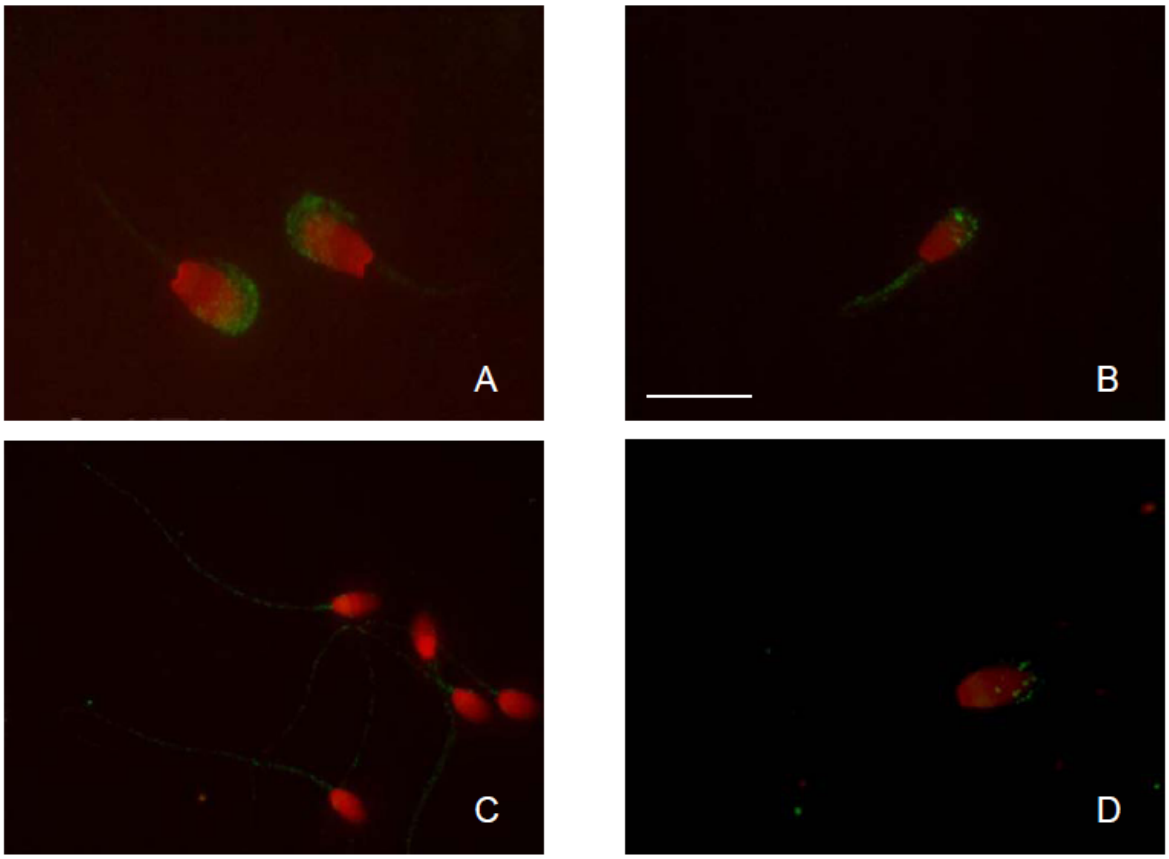

Figure 22. Representative photographs of GLUT 4 positivity in dog (A), boar (B), stallion (C) and donkey (D) spermatozoa. (Bar 10 μm)

Immunolocalization of GLUT 5

The fructose transporter showed the most constant localization among boar, dog and stallion sperm cells, in all of which it was localized in the acrosome membrane and in the tail. In most of stallion sperm cells an equatorial line was also present. In donkey spermatozoa it was detected in the tail, particularly in the principal piece, with the exclusion of the end and midpiece, and in the AM.

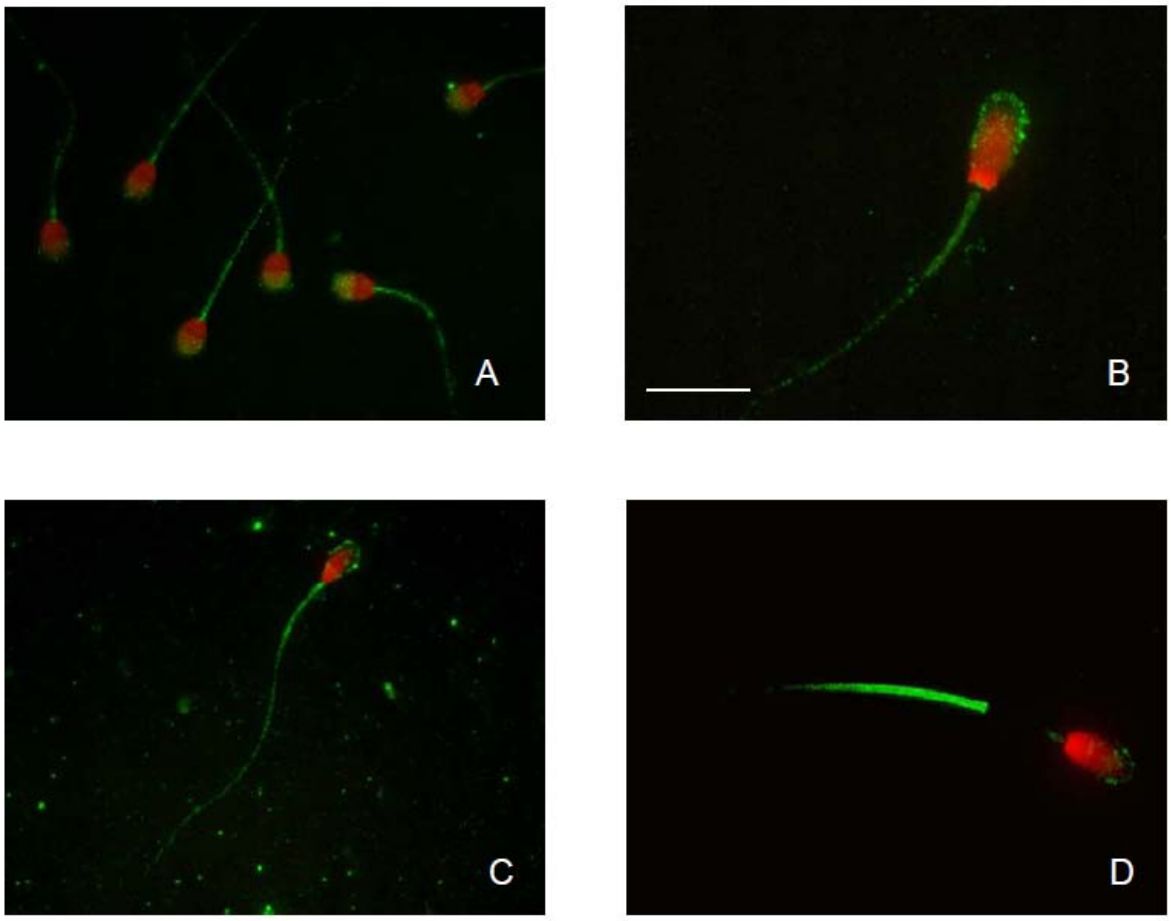

Figure 23. Representative photographs of GLUT 5 distribution in dog (A), boar (B), stallion (C) and donkey (D) spermatozoa. (Bar 10 µm)

Discussion

Data concerning viability were used to perform a pre-screening of the samples, in order to work in the best and most physiological conditions.

As for donkey semen parameters such as sperm motility, morphology and viability, they are consistent with those reported by Mirò et al. (2005), with some differences, especially in total volume and VAP, that could be due to the different breed of animals used in the experiments as well as to the breeding season period.

Angulo et al. (1998) demonstrated GLUT 1 expression in human, rat and bull spermatozoa and showed a cellular localization, similar to the horse and boar ones described in this study, at the level of the acrosome and in the principal piece of the sperm tail with the exclusion (total in rat and bull, partial in man) of the midpiece. Our findings clearly show other differences: the acrosomal positivity in boar and stallion is

not diffuse, but it is distributed in a spotted pattern and horse's cells present an evident equatorial line. In addition, dog GLUT 1 shows a clear positivity in the external acrosomal membrane and not a distributed positivity.

As for GLUT 2, our results show that its distribution pattern is similar in boar and rat and in man, dog and stallion, respectively (Angulo et al., 1998), while bull is different, being its positivity evident in the sperm head and mid-piece.

We could find some similarities in GLUT 3 distribution only between man, horse and boar spermatozoa, while rat ones are immunoreactive only in the tail, and bull ones are positive in the sperm head and mid-piece (Angulo et al., 1998).

As for boar spermatozoa, our findings are consistent with those by Medrano et al. (2006), who confirmed the peculiar aspect of the acrosomal positivity as a cluster of immunoreactive complexes; the physiological role of this distribution is not clear yet, even if others (Carruthers, 1990) report a tendency of glucose transporters to form self-associations. Furthermore, GLUT 3 position is strongly correlated to hexokinase distribution in cytoplasm (Medrano et al., 2006): being that glycolytic enzyme binds to tail's fibrous sheath in mouse sperm cells (Krifalusi et al., 2006), we may hypothesize that GLUT 3 distribution is strictly related to enzymes involved in glycolytic chain, particularly as concerning their localization in the sperm tail. This hypothesis is strengthened by the results by Medrano et al. (2006), demonstrating the importance of hexokinase I as a regulatory factor for glycolysis in boar sperm cells, together with the presence of any hexose transporters. According to this hypothesis, we may suppose a different role played by the different GLUTs in regulating the entrance of hexoses in the sperm cell, with particular attention to fructose and hexose transporters, and the metabolic pathways these substrates undergo in sperm cells of different mammalian species (Fernandez-Novell et al., 2004).

As for immunolocalization of GLUT 3 in dog fresh semen, our results differ from those by others (Rigau et al., 2002), who described a mid-piece immunoreactivity with exclusion of the end-piece. This discrepancy could be the consequence of the fixation technique utilized.

Medrano et al. (2006) demonstrated the presence of GLUT 3 not only on the sperm external acrosomal membrane, but also in some internal structures and suggested it could undergo an exposure on the cell membrane, similar to what GLUT 4 does in insulin sensitive tissues (Watson and Pessin, 2001). Our western blot results confirm

this possible intracellular localization, as we recorded a positive band in sonicated pellets, containing nuclei as well as other organelles of the sperm cell.

GLUT 4 has not been demonstrated in spermatozoa (Angulo et al., 1998), as only a weak or no positivity have been found by immunocytochemistry and no reactive bands by western blots. We also obtained very weak signals by immunocytochemistry, and only one weak reactive band in horse samples by western blot.

GLUT 5, the fructose transporter, is the most stable in localization among the three species; also the comparison in bull, rat and human spermatozoa localization described by Angulo et al. (1998) strengthens the hypothesis of a well conserved distribution of this transporter into sperm cells: only bull shows a very thick positivity in the end-piece of the tail and rat evidences a poor acrosomal positivity.

This study demonstrates for the first time both the presence and the localization of GLUTs 1, 2, 3 and 5 in donkey spermatozoa.

We focus the interest of data from donkey on the differences between horse and donkey, as they are members of the same genus and there are many differences in the localization of GLUTs. In donkey spermatozoa GLUT 1 is localized in the upper external part of the AM and in the tail, with the exception of the midpiece, which is poorly positive, while as already seen before, in stallion sperm it is localized in the tail, in the AM (with a spotted pattern) and in the equatorial line. GLUT 2 was found in the AM, tail and equatorial line of donkey spermatozoa, as in horse; in both species GLUT 3 is localized in the tail and in the AM. However, in donkey sperm cells it is localized in the apical external part of AM, the equatorial line is more evident and the midpiece has a fainter positivity; a common feature is the evident neck spot. Finally, GLUT 5 has the same MW in the two species, but the localization is different: in stallion it is localized in both tail and AM, while in donkey it is present only in the principal piece of the tail and in the AM.

GLUT 3 MW is different in the two species, being about 50 KDa in stallion and higher (65 KDa) in donkey. This difference could represent a starting point for future studies aimed at identifying possible molecular and phylogenetic differences between the two species.

GLUT 4 presented a low positive signal in immunofluorescence, especially in the tail, but no positive signal in western blotting analysis, as already reported for boar, dog and horse.

The presence and the peculiar localization of GLUT 5 in donkey spermatozoa rises another interesting point: as horse seminal plasma contains low rate of fructose (ranging from 2 to 7,6 mg/dl) (Garner and Hafez, 2008), we can hypothesize that donkey seminal plasma could contain a similar low concentration of this sugar. Two hypotheses could be advanced in order to explain the presence of GLUT 5, a fructose transporter, in a milieu lacking fructose:

- GLUT 5 could play an important role during the post-ejaculation life in the female genital tract, whose secretions could provide the substrate for this carrier and for sperm metabolism;
- this carrier transports other molecules than fructose, such as glucose (Sheperd, 1992).

GLUT 4 showed no specific signal in western blotting analysis, and very weak positivity in immunocytochemistry, so that we can suggest that this isoform is not present in mammalian sperm cells of the species we studied, thus confirming the results by Medrano et al. (2006) and those by Angulo et al. (1998)

We should report that GLUTs 1 and 2 were not found by Medrano and co-workers (2006) in boar spermatozoa.

Experiment 2

Evaluation of GLUTs re-localization after capacitation and acrosome reaction in boar, stallion and dog spermatozoa

Results

Assessment of capacitation and acrosome reaction

Capacitation rate and acrosome reaction (AR) were assessed in spermatozoa after incubation under capacitating condition and incubation with calcium ionophore A23187, in order to establish the capacitation and acrosome reaction rate of our samples.

As for stallion and dog, the capacitation pattern, observed with CTC staining method, was 21.9±1.9 % and 32.7±5 % respectively.

In boar semen capacitation rate was assessed on the basis of immunolocalization of Hsp 70 (see materials and methods), as it is a more precise and effective method. We found that 88.5% of sperm cells had a capacitated pattern; this high rate is due to the high sensibility of the test, that can also detect the very early changes in sperm membrane, as it is well described by Spinaci et al. (2005).

Acrosome reacted spermatozoa were 94.9±0.1 % in stallion, 87.2±2 % in boar and 83.1±3 % in dog.

Immunolocalization of GLUT1

After capacitation, boar and stallion spermatozoa didn't show any difference in either intensity or localization of the signal, while dog spermatozoa expressed a different localization of GLUT 1: in fact, acrosome membrane positivity clearly showed a relocalization of the signal, that spread from the upper external part of the acrosome to the whole acrosomal membrane.

AR didn't seem to exert any effect on sperm localization of GLUT 1, in all the species, except for the disappearance of the positivity in the acrosomal region, due to the removal of the acrosome.

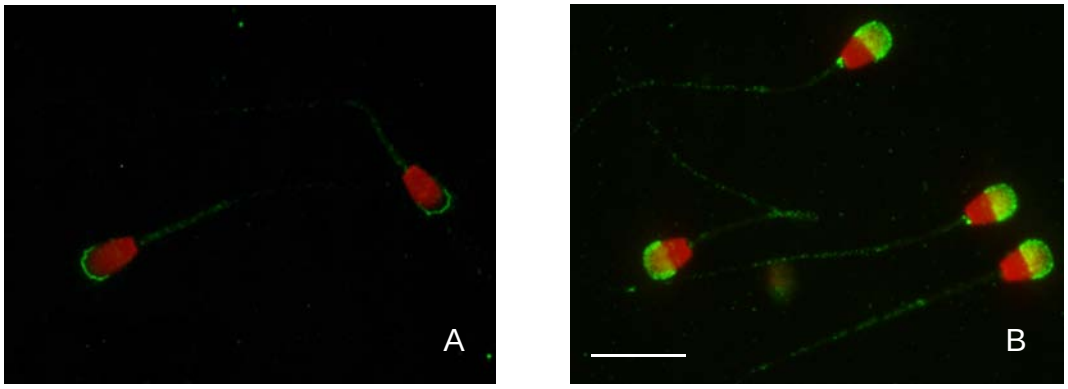

Figure 23. Representative immunolocalization of GLUT 1 in fresh (A) and capacitated (B) dog semen. (Bar 10 μm)

Immunolocalization of GLUT 2

No differences were recorded in boar and stallion spermatozoa after capacitation. In dog, the positivity changed a little bit: mid-piece positivity disappeared, while there was an increase in acrosomal positivity.

As for the acrosome reacted spermatozoa, no changes were recorded in any species, except for the disappearance of the acrosomal positivity.

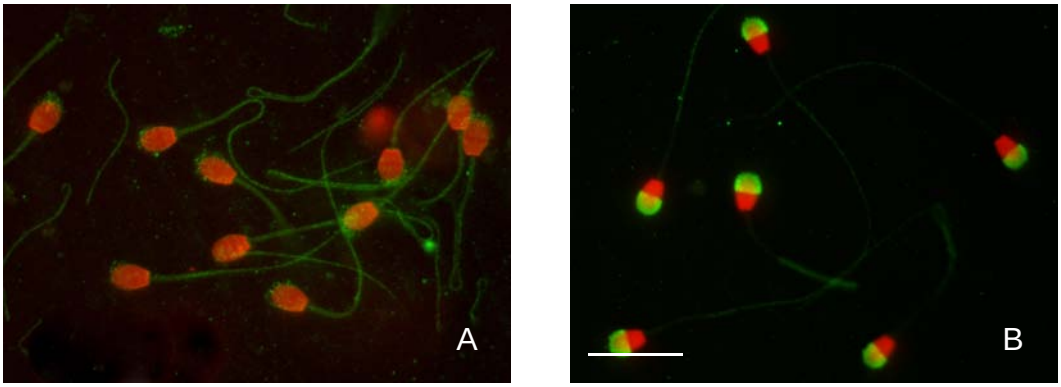

Figure 24. Representative photographs of GLUT 2 distribution in fresh (A) and capacitated (B) dog semen. (Bar 10 μm)

Immunolocalization of GLUT 3

As already seen for GLUTs 1 and 2, the capacitation induced modifications in GLUT 3 localization only in dog's spermatozoa. In fact, GLUT 3 positivity of capacitated dog spermatozoa was localized in the acrosome and, with a vivid and homogeneous signal, in the whole tail, so including the midpiece.
Acrosome reaction never caused changes in either intensity or distribution of GLUT 3 signal except for the disappearance of the acrosomal positivity .

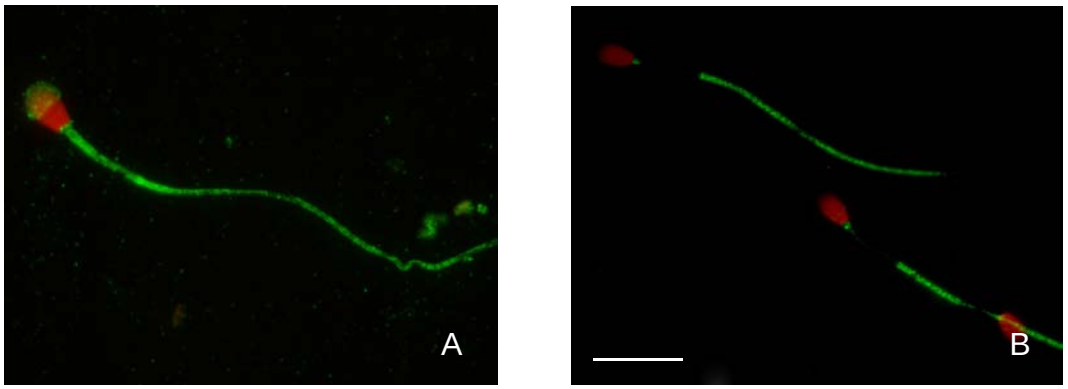

Figure 25. Representative photographs of GLUT 3 distribution in fresh (A) and capacitated (B) dog semen. (Bar 10 μm)

Immunolocalization of GLUT 5

After capacitation, a difference was detected only in canine spermatozoa: the intensity of tail positivity increased, while that of acrosome became fainter.
Also in the case of this GLUT, no significant modifications were observed in the three species when AR was induced, except for the disappearance of the acrosomal positivity.

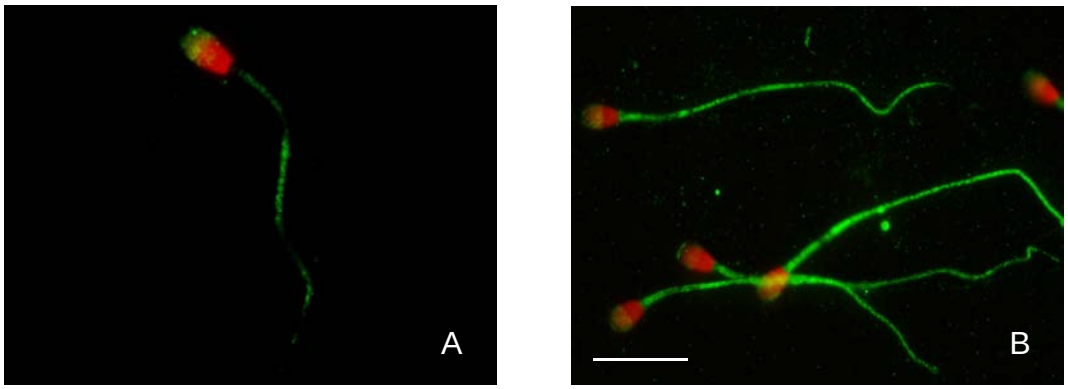

Figure 26. Representative photographs of GLUT 5 distribution in fresh (A) and capacitated (B) dog semen. (Bar 10 µm)

Discussion

As it is well-known, a rearrangement of the acrosomal membrane takes place after capacitation, involving both protein and lipid distribution; in addition, sperm cells change their motility pattern, from the so-called “activated pattern” to the hyper-activated motility (Yanagimachi, 2008) . We should also remember that during capacitation there is an overall activation of the cell function, as well as an activation of some intracellular modulators under the form of protein phosphorylation, that certainly requires more energy (see introduction).

As stated in the results, no relocalization was evident in boar and stallion spermatozoa, but only in dog sperm cells.

These results could be explained by two different hypotheses: a passive relocation of the proteins or their active relocation.

Capacitation could induce the relocation of some proteins that could be important for the next stages of sperm cell life, such as acrosomal reaction and penetration into the zona pellucida, and the translocation of the different GLUTs could be a passive consequence of the active movement of these proteins.

Alternatively, GLUTs could actively move depending on the position of the different metabolic pathways the hexoses undergo.

The differences between dog and boar hexose metabolism, described by others (Ballester et al., 2000; Rigau et al., 2002; Marin et al., 2003; Albarracìn et al., 2004; Fernandez-Novell et al., 2004; Medrano et al., 2006) could explain an active relocation of GLUTs in dog but not in boar and stallion spermatozoa.

In fact, the “dog-phenotype” sperm cells (Rodriguez-Gil, 2006) possess two different hexokinases, one of those is a high-km hexokinase (Fernandez-Novell, 2004), that permits to finely regulate glucose and fructose metabolization in the cell; there seems to be a glycolitic substrate circling in bull and dog spermatozoa (Hammested and Lardy, 1983; Rigau et al., 2002) that is linked to the regulation of hexoses metabolism; finally, in dog spermatozoa there is an active anabolic pathway, as glycogen synthesis (Ballester et al., 2000; Albarracìn et al., 2004). These are important peculiarities that could permit these spermatozoa to adapt to very diverse

metabolic and functional situations as capacitation, and the relocation of GLUTs could be an aspect of this metabolic system. Furthermore, dog spermatozoa remain in the female genital tract for a longer period than other species' sperm cells and this could lead to the necessity of a different metabolic strategy in energy management that could explain the relocation of GLUTs after capacitation.
Acrosomal-reacted spermatozoa do not present any difference in GLUTs position if compared with capacitated spermatozoa, except for a lack of positivity after both plasma and external acrosomal membrane removal. This is consistent with an effective localization of the main part of different GLUTs in the plasmatic membrane of the acrosomal region.
GLUT 4 wasn't analyzed in this study, as our previous data indicate that this protein is not actually present in mammalian sperm cells.

In conclusion, we discovered and described a relocalization mechanism of GLUTs in dog sperm cells, that does not seem to take place in boar and stallion spermatozoa. This translocation could be referred to a passive mechanism or to an active adaptation of the cell to new metabolic conditions. The induction of the acrosome reaction does not seem to affect the localization of GLUTs 1, 2, 3 and 5.

Experiment 3

Sex sorting procedure and stimulation with either IGF or insulin does not affect GLUT localization in boar spermatozoa

Results

Capacitation state of the cells was assessed, as already stated in the previous experiment, by immunolocalization of Hsp70. No significant differences were reported between capacitated (control group) and treated cells. In addition, difference between the acrosome reacted cells after capacitation and capacitation with INS and IGF were not significant.

Immunolocalization of GLUTs 1, 2, 3 and 5 didn't show any difference if compared with "normally" capacitated cells, as well as with fresh cells.

It is stated that sperm fluorcytometric sex sorting procedure determines the relocation of some proteins in boar spermatozoa, such as Hsp70 (Spinaci et al., 2006) and that it induces membrane suffering and damage. Our results demonstrate that it doesn't affect GLUT distribution among the cell membrane in boar spermatozoa; the only evident data are clearer positivity and strong signal.

Discussion

Insulin and insulin receptor have been shown to play an important role in reproductive function, both in gamete differentiation and maturation (Nakayama et al., 1999), as well as in sperm function (Shrivastav et al., 1989; Baccetti et al., 2002).

Some Authors described the presence of insulin mRNA in man and boar sperm, as well as the presence of the entire protein and its receptor (Andò and Aquila, 2005; Carpino, 2009). It has been stated that both insulin and leptin play a role in human sperm activity and metabolism, as they enhance hyperactivates motility, ROS production and acrosome reaction (Lampiao and du Plessis, 2008).

The regulatory action of insulin and IGF can be exerted by inducing a capacitation-like signaling, such as the MAPK stimulation via insulin receptor activation, that could lead to turn on the IP-3 signaling pathway (Andò and Aquila, 2005).

We studied GLUT distribution among the membrane in fresh and capacitated boar sperm cell and we did not find any difference between these two groups of cells, in both boar and stallion.

The stimulation with INS and IGF under capacitating condition does not exert any effect on GLUTs distribution in boar sperm cells, as it was expected from the results of capacitation.

We could affirm that GLUTs don't re-localize in boar sperm membrane after capacitation as well as after stimulation with metabolic hormones. It could be hypothesized that boar sperm cells don't need to re-localize its transporters to obtain a good capacitating status and that the distribution of these transporters can respond to the different necessities the cell undergoes after capacitation and acrosome reaction. In addition, we can assume that what has been hypothesized for dog spermatozoa, as a possible passive relocalization of GLUTs, does not take place in boar sperm due to a different membrane organization, that could be explained by the deep difference in metabolic status and "phenotype" of the two species cells (Medrano et al., 2006; Rodriguez-Gil., 2006).

For what concerns our results on sex sorted semen, the stresses spermatozoa undergo during the process don't seem to affect the membrane localization of GLUTs as assessed by immunofluorescence.

The flow cytometric technique induces a lot of different stress on sperm cells: in fact, spermatozoa are extremely diluted, then stained with Hoechst, then have to pass through a high voltage electromagnetic field and finally are thrown into the collecting tubes at a high velocity. All these passages markedly influence sperm viability, membrane integrity and protein distribution (Spinaci et al., 2006). Despite these findings, GLUTs are not affected by changes in their distribution after sex sorting process; only a clearer positivity and a more intense signal have been noted.

These two aspects are surely due to the intense dilution and washing the cells undergo before, during and after the sex sorting process, that contribute to clean the medium in which cells are suspended, as well as to clear cells themselves, by an intense membrane washing.

The fact that any protein, such as Hsp 70, undergo a relocation after sex sorting procedure could be related to simil-capacitation changes in the function of these proteins (Spinaci et al., 2006), that don't involve GLUTs, because, as we already

hypothesized, pig spermatozoa don’t need a relocation of these proteins to regulate their metabolic activity nor to improve their hexose uptake capacity.

General Discussion

The presence of the various GLUTs in sperm cells can represent an interesting point to be deepened, as it has been demonstrated (Ballester et al., 2000; Rigau et al., 2002; Medrano et al., 2006; Rodriguez-Gil, 2006) that there are some control points in hexose metabolism in sperm cells and GLUTs can represent a turning point in this control.

In fact, the presence and location of a high affinity hexokinase in dog (Fernandez-Novell et al., 2004) are an important discovery to differentiate sperm metabolism in various species, as this enzyme is not present in boar, whose metabolism is quite different from dog one and could be considered as a different metabolic phenotype (Rodriguez-Gil, 2006). It has been supposed that GLUTs can be a further control point in hexose utilization, as they can modulate the substrate influx into the cell. It is reasonable to think that this modulation is achieved by the presence of various isoforms in different cell compartments: as we demonstrated, each GLUT isoform has a peculiar localization in the sperm cell, that is quite different in the various species we considered. This could be due to the different metabolic strategy used by sperm cells to supply themselves with energy substrates, as well as to the possible co-localization of some enzymes involved in metabolic processes. Fructose and glucose entry into the cells is modulated by the different affinity of the transporters for these substrates: GLUT 1 is the most diffused transporter and its activity is specific for glucose, as for GLUT 3, which has a higher transport rate; GLUT 5 is the specific fructose transporter, while GLUT 2 is a fructose and glucose transporter. As demonstrated, these isoforms are present in dog, boar, horse and donkey spermatozoa, each one with its peculiar localization among the cell membrane and this finding can be easily related to the differences between species. Boar is the most “glycolitic” of the species that has been considered, and has a great diffusion of all the considered isoforms, with particular importance for GLUT 3 and GLUT 5. The presence of the high affinity glucose transporter GLUT 3 is quite easy to be understood, as glucose is the main substrate that can be found in seminal plasma (Garner and Hafez, 2008) and is the most “activating” one (Medrano et al., 2006), but the presence of high amount of fructose transporter GLUT 5 is not related with a specific physiological presence or function of this hexose. What’s more, the presence

of the different transporters, and particularly of GLUT 3 in the midpiece, can be related to an activation or hyperactivation of the mitochondrial function.

The presence and the different localization of GLUTs in horse and donkey spermatozoa are curious: the two species are, as we already reported, very near, but there are differences in GLUTs localization as well as in their molecular weight. The information about the metabolism of these two species spermatozoa are very scarce, and a deeper study could permit a better understanding of these differences. It is important to remember that these spermatozoa are the fastest among domestic species, and the support to such a motility should be provided by a strong energy producing apparatus, that is surely related with the presence of the various GLUT isoforms.

As for dog, it represents the more interesting finding of this work. We remember that this species has a particular metabolic phenotype that can be explained by its capacity to produce and accumulate glycogen, to regulate hexose substrates consume by pentose phosphate pathway (PPP) and finally to “economize” energy for a long period of time in which they can survive in the female genital tract.

Dog spermatozoa are the only ones that undergo changes in GLUTs location after incubation under capacitating conditions. We already furnished some explications for this phenomenon, that are strictly related to the molecular organization of sperm cell membrane: this relocalization takes place because of an active movement among the membrane or, on the other side, because of a movement of other proteins, that induces a passive translocation of GLUTs. The metabolic implication of this translocation, being it passive or active, is anyway pregnant of significance: we are in front of a cell that changes its membrane protein disposition after a functional change that involves metabolism, motility, protein and membrane status. It could be simply related to the change in membrane fluidity that occur after capacitation (Flesh and Gadella, 2005), but it is not clear why this process would involve GLUTs, while in other species, where the changes in membrane fluidity occur in the same manner, it doesn’t happen. It could be useful to find a direct link between the peculiar metabolism of dog spermatozoa and the “behavior” observed in GLUTs, as it could explain the regulation pathways under a new point of view, about which we can now only speculate.

At the same time we don’t really know why only dog spermatozoa undergo similar changes in GLUT distribution, as it is demonstrated that, at least in boar, there is a

reorganization of the lipidic surface of the cell (van Gestel et al., 2005) as a consequence of the exposition to bicarbonate or other capacitating conditions. Probably GLUTs are anchored to some structures beyond the membrane, that don't permit a relocation of these molecules. In boar only Sancho and co-workers (2007) found a relocalization of GLUT 3 but not of GLUT 5 after the freezing/thawing process, consistent in a loss of signal strength and a redistribution among the whole cell membrane.

Another argument raised by our investigations on GLUTs is that the spermatozoon can adapt to a different environment as the female genital tract. We didn't perform any in vivo trials, but we can speculate on the physiologic role of the presence of some GLUTs: is there a direct link between GLUTs presence and substrate availability in seminal plasma and female genital fluid? As we already evidenced, in horse and donkey there is a very low levels of fructose in the seminal plasma, but GLUT 5 is expressed (particularly in donkey), so it is likely they can take advantage of GLUT 5 presence in female genital tract. We should consider that the life period a spermatozoon spends in the female genital tract can be relatively short (bull, boar) or long (dog), and that it is important for the cell to maintain all its features to reach a complete capacitation and further acrosome reaction, as well as to perform fertilization.

These studies provide other proofs about the absence of GLUT 4 in mammalian sperm cells. Other authors (Angulo et al., 1998; Medrano et al., 2006) didn't find a positivity in their investigations on GLUT 4 in bull, human, rat and boar spermatozoa. We furnish further data on boar spermatozoa and new findings in horse, donkey and dog spermatozoa: the faint positivity we observed in immunofluorescence wasn't confirmed by western blot analysis, as no positivity or no specific band was detected in cell lysates.

A possible explanation of this phenomenon is that GLUT 4 is usually found in intracellular structures (Fukumoto et al., 1989) in insulin sensitive tissues, and spermatozoa don't have enough cytoplasm to contain such structures, that are not indispensable. In addition, sperm cells possess insulin receptor and (seem) to produce insulin (Andò and Aquila, 2005), but it is not stated the role insulin could exert in sperm activity: we can suppose it is not a typical action leading to increase glucose uptake, as GLUT 4 seems to be absent, but it can interfere in some mechanisms related to sperm function, as capacitation or acrosome reaction.

Experiments we carried out to stimulate spermatozoa with insulin or IGF in pig didn't induce any change in GLUT localization under capacitating conditions. We already discussed data concerning the lack of effect of the capacitating condition on GLUT localization in boar and stallion and we can add some considerations as for boar. In this species, in fact, the energy producing machinery works at top of its potential in fresh cells at a glucose concentration near to 5 μM (Fernandez–Novell et al., 2004) as hexokinase I, the main control point in sperm glycolitic chain, has this quality. We do not know what is the glucose consuming rate under capacitating conditions, but it can't be very far from that observed in fresh cells, as the enzymatic apparatus is the same, and there could be a difference into substrate fluxes, more than an augmented activity of the various enzymes. In addition, our capacitating medium has a higher glucose concentration than that considered the best for sperm functioning, and the producing activity cannot be possibly overstressed by different stimuli as insulin or IGF. These observations concerning cell metabolic capacity could furnish a functional explanation of the stability of GLUTs distribution among the cell membrane during and after capacitation.

The last observation is that GLUTs demonstrate to have a very stable disposition even after a membrane stressing process as cytofluorimetric sex sorting in boar spermatozoa. In fact, it has been reported (Spinaci et al., 2006) that Hsp 70 undergoes a simil-capacitating relocalization after the sex sorting process. On the other side we showed that GLUTs are very stable and don't move among the cell membrane, thus supporting the hypothesis by Sancho and co-workers (2007) that almost some of these proteins could be anchored in sub-membranal structures, and that only a very high membrane mechanical and functional stress could affect their location and presence.

Conclusions

Our studies permit these conclusive remarks:

- GLUTs 1, 2, 3, and 5 are present in boar, dog, stallion and donkey spermatozoa; their localization among the cell membrane has been determined and described;
- GLUT 4 does not seem to be present in boar, stallion, dog and donkey spermatozoa, as only very weak signals were detected in immunofluorescence and no positive or specific signal has been detected in western blotting analysis;
- Capacitation process induces changes in dog spermatozoa GLUTs distribution, but not in stallion and boar ones; the mechanism lying behind this finding is not clear, and it should be deepened;
- Our investigation in boar spermatozoa didn't show any change in GLUT distribution either under capacitating process or after stimulation with insulin and IGF;
- Sex sorting process does not interfere with GLUTs localization.

Bibliography

Aalberts J. G., Mann T., Polge C. (1961). Metabolism of boar semen in relation to sperm motility and survival. J Reprod Fertil **2** 42-53.

Abou-haila A., Tulsiani D. R. P. (2009) Signal transduction pathways that regulate sperm capacitation and the acrosome reaction. Arch Biochem Biophys **485(1)** 72-81.

Albarracín J. L., Fernández-Novell J. M., Ballester J., Rauch M. C., Quintero-Moreno A., Peña A., Mogas T., Rigau T., Yañez A., Guinovart J. J., Slebe J. C., Concha I. I., Rodríguez-Gil J. E. (2004) Gluconeogenesis-linked glycogen metabolism is important in the achievement of in vitro capacitation of dog spermatozoa in a medium without glucose. Biol Reprod. **71(5)** 1437-1445.

Al-Hasani H., Kunamneni R. K., Dawson K., Hinck C. S., Muller-Wieland D., Cushman S. W. (2002) Roles of the N- and C-termini of GLUT4 in endocytosis. J Cell Sci **115** 131–140.

Andò S., Aquila S. (2005) Arguments raised by the recent discovery that insulin and leptin are expressed in and secreted by human ejaculated spermatozoa. Mol Cell Endocr. **245** 1-6.

Angulo C., Rauch M. C., Droppelmann A., Reyes A. M., Slebe J. C., Delgado-Lopez F., Guaiquil V. H., Vera J. C., Concha I. I. (1998) Hexose transporters expression and function in mammalian spermatozoa: cellular localization and transport of hexoses and vitamin C. Biochem Cell Biol **71** 189-203.

Baccetti B., La Marca A., Piomboni P., Capitani S., Bruni E., Petraglia F., De Leo V. (2002) Insulin-dependent diabetes in men is associated with hypothalamo-pituitary derangement and with impairment in semen quality. Hum Reprod **17** 2673–2677.

Ballester J., Fernandez-Novell J. M., Ruttlant J., Garcia-Rocha M., Palomo M. J., Mogas T., Peña A., Rigau T., Guinovart J. J., Rodriguez-Gil J. E.(2000) Evidence for

a functional glycogen metabolism in mature mammalian spermatozoa. Mol Reprod Dev **56** 207-219.

Birnbaum M. J., Haspel H. C., Rosen O. M., (1986) Cloning and characterization of a cDNA encoding the rat brain glucose transporter protein. Proc. Natl. Acad. Sci. USA **83** 5784-5788.

Braun R. E. (2001) Packaging paternal chromosomes with protamine. Nat Genet **28** 10-12.

Breitbart H., Spungin B. (1997) The biochemistry of the acrosome reaction. Mol Hum Reprod **3(3)** 195–202.

Breitbart H:, Naor Z. (1999) Protein kinases in mammalian sperm capacitation and the acrosome. Rev Reprod **4** 151–159.

Breitbart H, Cohen G, Rubinstein S. (2005) Role of actin cytoskeleton in mammalian sperm capacitation and the acrosome reaction. Reproduction **129(3)** 263-268.

Breitbart H., Rubinstein S., Etkovitz N. (2006) Sperm capacitation is regulated by the crosstalk between protein kinase A and C. Mol Cell Endocrinol **252** 247–249.

Breitbart H, Rotman T, Rubinstein S, Etkovitz N. (2009) Role and regulation of PI3K in sperm capacitation and the acrosome reaction. Mol Cell Endocrinol In press.

Brener E., Rubinstein S., Cohen G., Shternall K., Rivlin J., Breitbart H. (2003) Remodeling of the actin cytoskeleton during mammalian sperm capacitation and acrosome reaction. Biol Reprod **68** 837–845.

Brüssow K. P., Ratky J., Rodriguez-Martinez H. (2008) Fertilization and early embryonic development in the porcine fallopian tube. Reprod Dom Anim **43 (Suppl. 2)** 245–251.

Burant C. F., Takeda J., Brot-Laroche E., Bell G. I., Davidson N. O. (1992) Fructose transporter in human spermatozoa and small intestine is GLUT5. J Biol Chem **267(21)** 14523-14526.

Cabello-Agueros J. F., Hernandez-Gonzalez E. O., Mujica A. (2003) The role of F-actin cytoskeleton-associated gelsolin in the guinea pig capacitation and acrosome reaction. Cell Motil Cytoskeleton **56** 94–108.

Carayannopoulos M. .O, Chi M. M., Cui Y., Pingsterhaus J. M., McKnight R. A., Mueckler M., Devaskar S. U., Moley K.H. (2000) GLUT8 is a glucose transporter responsible for insulin-stimulated glucose uptake in the blastocyst. Proc Natl Acad Sci USA **97** 7313–7318.

Carayannopoulos M. O., Schlein A., Wyman A., Chi M., Keembiyehetty C., Moley K. H. (2004) GLUT9 is differentially expressed and targeted in the preimplantation embryo. Endocrinology **145(3)** 1435-1443.

Carpino A., V. Rago V., Guido C., Casaburi I., Aquila S. (2009) Insulin and IR-β in pig spermatozoa: a role of the hormone in the acquisition of fertilizing ability. Int J Androl In press.

Carruthers A. (1990) Facilitated diffusion of glucose Physiol Rev **70(4)** 1135-76.

Castellani-Ceresa L., Mattioli M., Radaelli G., Barboni B., Brivio M. F. (1993) Actin polymerization in boar spermatozoa: fertilization is reduced with use of cytochalasin D. Mol Reprod Dev **36** 203–211.

Chandler J. D., Williams E. D., Slavin J. L., Best J. D., Rogers S. (2003) Expression and localization of GLUT1 and GLUT12 in prostate carcinoma. Cancer **97** 2035–2042.

Christensen P., Whitfield C. H., Parkinson T. J. (1996) In vitro induction of acrosome reaction in stallion spermatozoa by heparin and A23187. Theriogenology **45** 1201-1210.

Cohen G., Rubinstein S., Gur Y., Breitbart H. (2004) Crosstalk between protein kinase A and C regulates phospholipase D and F-actin formation during sperm capacitation. Dev Biol **267** 230–241.

Cross N. L., Watson S. K. (1994) Assessing acrosomal status of bovine sperm using fluorescinated lectins. Theriogenology **42** 89-98.

Dacheux, J. L., Belghazi, M., Lanson, Y. and Dacheux, F. (2006) Human epididymal secretome and proteome. Mol Cell Endocrinol **250(1–2**) 36–42.

Davson H. (1964) A textbook of general physiology. 3rd Ed. J and A Churchill Ltd, London.

Dawson P. A., Mychaleckyj J. C., Fossey S. C., Mihic S. J., Craddock A. L., Bowden D. W. (2001) Sequence and functional analysis of GLUT10: a glucose transporter in the Type 2 diabetes-linked region of chromosome 20 12–13. Mol Genet Metab **74** 186–199.

De Lamirande E., Gagnon C. (1995) Impact of reactive oxygen species on spermatozoa: a balancing between beneficial and detrimental effects. Hum Reprod **10** 15–21.

De Lamirande E., O'Flaherty C. (2008) Sperm activation: Role of reactive oxygen species and kinases. Biochim Biophys Acta **1784(1)** 106-115.

Diez-Sampedro A., Lostao M. P., Wright E. M., Hirayama B. A. (2000) Glycoside binding and translocation in Na(_)-dependent glucose co-transporters: comparison of SGLT1 and SGLT3. J Membr Biol **176** 111–117.

Diez-Sampedro A., Eskandari S., Wright E. M., Hirayama B. A. (2001) Na^+-to sugar stoichiometry of SGLT3. Am J Physiol Renal Physiol **280** F278–F282.

Diez-Sampedro A., Hirayama B. A., Osswald C., et al. (2003) A glucose sensor hiding in a family of transporters. Proc Natl Acad Sci U S A. 100 11753–11758.

Doege H., Bocianski A., Joost H. G., Schürmann A. (2000) Activity and genomic organization of human glucose transporter 9 (GLUT9), a novel member of the family of sugar-transport facilitators predominantly expressed in brain and leucocytes. Biochem J **358(Pt 3)** 791-792.

Doege H., Schürmann A., Bahrenberg G., Brauers A., Joost H. G. (2000b) GLUT8, a novel member of the sugar transport facilitator family with glucose transport activity. J Biol Chem **275**(**21**) 16275-16280.

Doege H., Bocianski A., Scheepers A., Axer H., Eckel J., Joost H. G., Schürmann A., (2001) Characterization of human glucose transporter (GLUT) 11 (encoded by SLC2A11), a novel sugar-transport facilitator specifically expressed in heart and skeletal muscle. Biochem. J. **359**(**Pt 2**) 443-449.

Dröge W. (2001) Free radicals in the physiological control of cell function. Physiol Rev **82** 47–95.

Eddy E. M., Toshimori K., O'brien D. A. (2003) Fibrous Sheath of Mammalian Spermatozoa. Microsc Res Tech **61(1)** 103-15.

Fawcett D. W. (1957) The structure of mammalian spermatozoa. Intl Rev Cytology **7** 195-234.

Fernandez-Novell J. M., Ballester J., Medrano A., Otaeguic P. J., Rigau T., Guinovart J. J., Rodriguez-Gil J. E. (2004) The presence of a high-Km hexokinase activity in dog, but not in boar, sperm. FEBS letters **570** 211-216.

Flesch F. M., Gadella B. M. (2000) Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochim Biophys Acta **1469(3)** 197-23.

Folgero T., Bertheussen K., Lindal S., Torbergsen T., Oian P. (1993) Mitochondrial disease and reduced sperm motility. Human Reprod **8** 1863–1868.

Ford W. C. L. (2006) Glycolysis and sperm motility: does a spoonful of sugar help the flagellum go round? Hum Reprod Update **12(3)** 269-274.

França L. R., Avelar G. F., Almeida F. L. (2005). Spermatogenesis and sperm transit through the epididymis in mammals with emphasis on pigs. Theriogenology. **63(2)** 300-318.

Fukumoto H., Seino S., Imura H., Seino Y., Eddy R. L., Fukushima Y., Byers M. G., Shows T. B., Bell G. I. (1988) Sequence, tissue distribution, and chromosomal localization of mRNA encoding a human glucose transporter-like protein. Proc. Natl. Acad. Sci. U.S.A. **85**(**15**) 5434-5438.

Fukumoto H., Kayano T., Buse J. B., Edwards Y., Pilch P. F., Bell G. I., Seino S. (1989) Cloning and characterization of the major insulin-responsive glucose transporter expressed in human skeletal muscle and other insulin-responsive tissues. J Biol Chem **264(14)** 7776-7779.

Gadella B. M. (2008) Sperm membrane physiology and relevance for fertilization. Anim Reprod Scie **107** 229–236.

Gadella B. M. (2008b) The assembly of a zona pellucida binding protein complex in sperm. Reprod Domest Anim **43(5)** 12-19.

Galantino-Homer H.L., Florman H.M., Storey B.T., Dobrinski I., and Kopf G.S. (2004) Bovine sperm capacitation: assessment of phosphodiesterase activity and intracellular alkalinization on capacitation-associated protein tyrosine phosphorylation. Mol Reprod Dev **67** 487-500.

Garner D. L., Hafez E. S. E. (2008) Spermatozoa and seminal plasma. In: Hafez B., Hafez E. S. E. (eds), Reproduction in Farm Animals, 7th edn. Lippincott Williams and Wilkins PA, USA pp. 96–109.

Gaster M., Handberg A., Schürmann A., Joost H. G., Beck-Nielsen H., Schroder H. D. (2004) GLUT11, but not GLUT8 or GLUT12, is expressed in human skeletal muscle in a fibre type-specific pattern. Pflugers Arch Eur J Physiol **448** 105–113.

Glander H. J., Dettmer D. (1978) Monosaccharide transport across membranes of human spermatozoa II. Basic properties of D-fructose and D-glucose uptake by spermatozoa. Andrologia **10** 273–277.

Glander H. J., Dettmer D. (1978b) Monosaccharide transport across membranes of human spermatozoa II. Basic properties of D-fructose and D-glucose uptake. Andrologia **10** 273–277.

González-Fernández L., Ortega-Ferrusola C., Macias-Garcia B., Salido G. M., Peña F. J., Tapia J. A. (2009) Identification of protein tyrosine phosphatases and dual-specificity phosphatases in mammalian spermatozoa and their role in sperm motility and protein tyrosine phosphorylation. Biol Reprod **80(6)** 1239-1252.

Gravance C. G., Garner D. L., Baumber J., Ball B. A. (2000) Assessment of equine sperm mitochondrial function using JC-1. Theriogenology **53(9)** 1691-1703.

Haber R. H., Wienstein S. P., O'Boyle E., Morgello S. (1993) Tissue distribution of the human GLUT 3 glucose transporter. Endocrinology **132-6** 2538-2543.

Hallap T., Nagy S., Jaakma U., Johannisson A., Rodríguez-Martínez H. (2005). Mitochondrial activity of frozen-thawed spermatozoa assessed by MitoTracker Deep Red 633. Theriogenology **63** 2311–2322.

Hammersted R. H., Lardy H. A. (1983) The effects of substrate cycling on the ATP yield of sperm glycolysis. J Biol Chem **258** 8759–8768.

Harayama, H., Magargee, S., Kunze, E., Shidara, O., Iwamoto, E., Arikawa, S., Miyake, M., Kato, S. and Hammerstedt, R. (1999) Changes in epididymal protein

anti-agglutinin on ejaculated boar spermatozoa during capacitation in vitro. Reprod Fertil Dev **11** 193–199.

Harrison R. A. P. (2004) Rapid PKA-catalysed phosphorylation of boar sperm proteins induced by the capacitating agent bicarbonate. Mol Reprod Dev **67** 337–352.

Harrison R. A. P., Gadella B. M. (2005) Bicarbonate-induced membrane processing in sperm capacitation. Theriogenology **63** 342-351.

Hebert D. N., Carruthers A. (1991) Cholate-solubilized erythrocyte glucose transporters exist as a mixture of homodimers and homotetramers. Biochemistry **30(19)** 4654-4658.

Hediger M. A., Turk E., Wright E. M. (1989) Homology of the human intestinal Na^+/glucose and Escherichia coli Na^+/proline cotransporters. Proc Natl Acad Sci USA **86** 5748–5752.

Holman G. D., Kozka I. J., Clark A. E., Flower C. J., Saltis J., Habberfield A. D., Simpson I. A., Cushman S. W. (1990) Cell surface labeling of glucose transporter isoform GLUT4 by bis-mannose photolabel. Correlation with stimulation of glucose transport in rat adipose cells by insulin and phorbol ester. J Biol Chem **265** 18172–18179.

Johnson L. A., Pinkel D. (1986) Modification of a laser-based flow cytometer for high resolution DNA analysis of mammalian spermatozoa. Cytometry **7** 268 –273.

Johnson L. A. (1991) Sex preselection in swine: altered sex ratios in offspring following surgical insemination of flow sorted X- and Y bearing sperm. Reprod Dom Anim **26** 309 –314.

Johnson L. A., Welch G. R. (1999) Sex pre-selection: high-speed flow cytometric sorting of X and Y sperm for maximum efficiency. Theriogenology **52** 1323 –1341.

Jones A. R. and Connor D. E. (2000) Fructose metabolism by mature boar spermatozoa Reprod Fertil Dev **12** 355–359.

Joost H. G., Thorens B. (2001) The extended GLUT-family of sugar/polyol transport facilitators: nomenclature, sequence characteristics, and potential function of its novel members (review). Mol Membr Biol **18(4)** 247-256.

Jung, C.Y., Carlson, L.M., Whaley, D.A. (1971) Glucose transport carrier activities in extensively washed human red cell ghosts. Biochim Biophys Acta **241** 613-627.

Kasahara M., Hinkle P. C. (1977) Reconstitution and purification of the D-glucose transporter from human erythrocytes. J Biol Chem **252(20)** 7384-7390.

Kayano T., Fukumoto H., Eddy R. L., Fan Y. S., Byers M. G., Shows T. B., Bell G. I. (1988) Evidence for a family of human glucose transporter-like proteins: sequence and gene localization of a protein expressed in fetal skeletal muscle and other tissues. J Biol Chem. **263** 15245–15248.

Kayano T., Burant C. F., Fukumoto H., Gould G. W., Fan Y. S., Eddy R. L., Byers M. G., Shows T. B., Seino S., Bell G. I. (1990) Human facilitative glucose transporters. Isolation, functional characterization, and gene localization of cDNAs encoding an isoforms (GLUT5) expressed in small intestine, kidney, muscle, and adipose tissue and an unusual glucose transporter pseudogenelike sequence (GLUT6). J Biol Chem **265** 13276–13282.

Kim Y. H., Haidl G., Schaefer M., Egner U., Mandal A., Herr J. C. (2007). Compartmentalization of a unique ADP/ATP carrier protein SFEC (Sperm Flagellar Energy Carrier, AAC4) with glycolytic enzymes in the fibrous sheath of the human sperm flagellar principal piece. Dev Biol. **302(2)** 463-76.

Kretser D. M. and Kerr J. B. (1988). The cytology of the testis. In: The physiology of reproduction. Vol. 1 Ed Knobil E., Neil J. D. et al. Raven Press, Ltd. New York.

Krifalusi M., Miki K., Magyar P. L., O'Brien D. (2006). Multiple glycolytic enzymes are tightly bound to the fibrous sheat of mouse spermatozoa. Biol Reprod **75** 270-278.

Lampiau F., du Plessis S. (2008) Insulin and leptin enhance human sperm motility, acrosome reaction and nitric oxide production. Asian J Androl **10(5)** 799-807.

Lardy H. A. and Phillips P. H. (1941) The interrelation of oxidative and glycolytic processes as sources of energy for bull spermatozoa. Am J Physiol **133** 602-609.

Lardy H. A., Hansen R. G., Phillips P. H. (1945) The metabolism of bovine epididymal spermatozoa. Arch Biochem **6** 41-51.

Lardy H. A., Winchester B., Phillips P.H. (1945b) The respiratory metabolism of ram spermatozoa. Arch Biochem **6** 33-40.

Lemieux M. J., Song J., Kim M. J., Huang Y., Villa A., Auer M., Li X. D., Wang D. N. (2003) Three-dimensional crystallization of the Escherichia coli glycerol-3-phosphate transporter: a member of the major facilitator superfamily. Protein Sci **12(12)** 2748-56.

Li Q., Manolescu A., Ritzel M., Yao S., Slugoski M., Young J. D., Chen X. Z., Cheeseman C. I. (2004) Cloning and functional characterization of the human GLUT7 isoform SLC2A7 from the small intestine. Am. J. Physiol. Gastrointest. Liver Physiol. **287**(**1**) G236-242.

Lillie F.R. (1919) Problems of Fertilization. University of Chicago Press, Chicago.

Lisinski I., Schürmann A., Joost H. G., Cushman S. W., Al-Hasani H. (2001) Targeting of GLUT6 (formerly GLUT9) and GLUT8 in rat adipose cells. Biochem J **358** 517–522.

Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J. (1951) Protein measurement with the folin phenol reagent. J Biol Chem **193** 265–75.

Malmgren L., (1997) Assessing the quality of raw semen: a review. Theriogenology **48** 523–530.

Mann T. (1946) Studies on the metabolism of semen. 3. Fructose as a normal constituent of seminal plasma. Site of formation and function of fructose in semen. Biochem J **40** 481-491.

Mann T. (1974) Secretory function of the prostate, seminal vesicle and other male accessory organs of reproduction. J Reprod Fertil **37** 179-188.

Mann T. (1975) Biochemistry of stallion semen. J Reprod Fertil Suppl **23** 47-52.

Marin S., Chiang K., Bassilian S., Lee W. P., Boros L. G., Fernandez Novell J. M., Centelles J. J., Medrano A., Rodriguez-Gil J. E., Cascante M. (2003) Metabolic strategy of boar spermatozoa revealed by a metabolomic characterization. FEBS Letters **554** 342-346.

McLeod J. (1941) The metabolism of human spermatozoa. Am J Physiol **132** 193-201.

McLeod J. (1943) The role of oxygen in the metabolism and motility of human spermatozoa. Am J Physiol **138** 512-518.

McPartlin L A , Littell J., Mark E., Nelson J. L., Travis A. J., Bedford-Guaus S. J. (2008) A defined medium supports changes consistent with capacitation in stallion sperm, as evidenced by increases in protein tyrosine phosphorylation and high rates of acrosomal exocytosis. Theriogenology **69(5)** 639-650.

McPartlin M. A., Suarez S. S., Czaya C. A., Hinrichs K., Bedford-Guaus S. J. (2009) Hyperactivation of stallion sperm is required for successful in vitro fertilization of equine oocytes. Biol Reprod **81(1)** 199-206.

Medrano A., Fernández-Novell J. M., Ramió L., Alvarez J., Goldberg E., Montserrat Rivera M., Gunivart J. J., Rigau T., Rodríguez-Gil J. E. (2006b) Utilization of citrate

and lactate through a lactate dehydrogenase and ATP-regulated pathway in boar spermatozoa. Mol Reprod Dev **73** 369-378.

Medrano A., García-Gil N., Ramió L., Montserrat Rivera M., Fernández-Novell J. M., Ramírez A., Peña A., Dolors Briz M., Pinart E., Concha I. I., Bonet S., Rigau T., Rodríguez-Gil J. E. (2006) Hexose-specificity of hexokinase and ADP-dependence of pyruvate kinase play important roles in the control of monosaccharide utilization in freshly diluted boar spermatozoa. Mol Reprod Dev **73(9)** 1179-1194.

Medrano A., Fernández-Novell J. M., Ramió L., Alvarez J., Goldberg E., Montserrat Rivera M., Gunivart J. J., Rigau T., Rodríguez-Gil J. E. (2006b) Utilization of citrate and lactate through a lactate dehydrogenase and ATP-regulated pathway in boar spermatozoa. Mol Reprod Dev **73** 369-378.

Miki K. (2007) Energy metabolism and sperm function. Soc Reprod Fertil Suppl **65** 309-325.

Miki K., Qu W., Goulding E. H., Willis W. D., Bunch D. O., Strader L. F., Perreault S. D., Eddy E. M., O'Brien D. A. (2004) Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. Proc Natl Acad Sci USA **101(47)** 16501-16506.

Mirò J., Lobo V., Quintero-Moreno A., Medrano A., Peña A., Rigau T. (2005). Sperm motility patterns and metabolism in Catalonian donkey semen. Theriogenology **63** 1706–1716.

Muekler M., Caruso C., Balwin S. A., Panico M., Blench I., Morris H. R., Allard W. J., Lienhard G. E., Lodish H. F. (1985) Sequence and structure of a human glucose transporter. Science **229(4717)** 941-5.

Mueckler M. (1994) Facilitative glucose transporters. Eur J Biochem **219(3)** 713-25.

Mukai C., Okuno M. (2004) Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. Biol Reprod **71** 540–547.

Nakamura M., Okinaga S., Arai K. (1987) Studies of metabolism of round spermatide: Cytochalasin B binding to cell membrane in relation to glucose transport. Andrologia **19** 178-182.

Nakayama Y., Yamamoto T., Abe S. I. (1999) IGF-I, IGF-II and insulin promote differentiation of spermatogonia to primary spermatocytes in organ culture of newt testes. Int J Dev Biol **43** 343–347.

Orci L., Unger R. H., Ravazzola M., Ogawa A., Komiya I., Baetens D., Lodish H. F., Thorens B. (1990) Reduced beta-cell glucose transporter in new onset diabetic BB rats. J Clin Invest **86(5)** 1615-22.

Parks J.K., Graham J.K. (1992) Effects of cryopreservation procedures on sperm membranes. Theriologenology **38** 209-222.

Peña F. J., Rodríguez Martínez H., Tapia J. A., Ortega Ferrusola C., González Fernández L., Macías García B. (2009). Mitochondria in mammalian sperm physiology and pathology: a review. Reprod Domest Anim **44(2)** 345-9.

Pessino A., Hebert D. N., Woon C. W., Harrison S. A., Clancy B. M., Buxton J. M., Carruthers A., Czech M. P. (1991) Evidence that functional erythrocyte-type glucose transporters are oligomers. J Biol Chem **266(30)** 20213-20217.

Peterson. R. N. and Freund. M. (1975) in Human Semen and fertility regulation in men. Hafez E. S. E. ed pp 176-186, Mosby, St Louis.

Peterson R. N., Bundman D., Freund M. (1977) Binding of cytochalasin B to hexose transport sites on human spermatozoa and inhibition of bindings by purines. Biol Reprod **17** 198-206.

Phay J. E., Hussain H. B., PhD, Moley J. F. (2000) Strategy for identification of novel glucose transporter family members by using Internet-based genomic databases. Surgery **128 (6)** 946-951.

Rathi R., Colenbrander B., Bevers M. M., Gadella B. M. (2001) Evaluation of in vitro capacitation of stallion spermatozoa. Biol Reprod **65** 462-470.

Redenz E. (1933). Über den spaltingsstoffwechsel der säugertierspermatozoen in zusammenhang mit der beweglichkeit. Biochem. Zeitschr. **257** 234-241.

Rigau T., Farrè M., Ballester J., Mogas T., Peňa A., Rodrìguez-Gil J.E. (2001) Effects of glucose and fructose on mobility patterns of dog spermatozoa from fresh ejaculates. Theriogenology **56** 801-815.

Rigau T., Rivera M., Palomo M. J., Fernández-Novell J. M., Mogas T., Ballester J., Peña A., Otaegui P. J., Guinovart J. J., Rodríguez-Gil J. E. (2002) Differential effects of glucose and fructose on hexose metabolism in dog spermatozoa. Reproduction **123(4)** 579-591.

Rodriguez-Gil J. E. (2006) Mammalian sperm energy resources management and survival during conservation in refrigeration. Reprod Domest Anim (**41 Suppl 2)** 11-20.

Rodriguez-Martinez H. (2007) Role of the oviduct in sperm capacitation. Theriogenology **68** 138–146

Rogers B. J., Bastias C., Coulson R. L., Russell L. D. (1989) Cytochalasin D inhibits penetration of hamster eggs by guinea pig and human spermatozoa. J Androl **10** 275–282.

Rogers S., Macheda M. L., Docherty S. E., Carty M. D., Henderson M., Soeller W. C., Gibbs E. M., James D. E., Best J. D. (2002) Identification of a novel glucose transporter-like protein GLUT 12. Am. J Physiol Endocrinol Metab **282**(**3**) E733-738.

Rogers S, Docherty SE, Slavin JL, Henderson MA, Best JD. (2003) Differential expression of GLUT12 in breast cancer and normal breast tissue. Cancer Lett **193** 225–233.

Rota A., Peña A. I., Linde-Forsberg C., Rodriguez-Martinez H., (1999) In vitro capacitation of fresh chilled and frozen-thawed dog spermatozoa assessed by the chlortetracycline assay and changes in motility patterns. Anim Reprod Sci **57** 199-201.

Ruiz-Pesini E., Díez-Sánchez C., López-Pérez M. J., Enríquez J. A. (2007) The role of the mitochondrion in sperm function: is there a place for oxidative phosphorylation or is this a purely glycolytic process? Curr Top Dev Biol **77** 3-19.

Sancho S., Casas I., Ekwall H., Saravia F., Rodriguez-Martinez H., Rodriguez-Gil J.E., Flores E., Pinart E., Briz M., Garcia-Gil N., Bassols J., Pruneda A., Bassaleu E., Yeste M., Bonet S. (2007) Effects of cryopreservation on semen quality and the expression of sperm membrane hexose transporters in the spermatozoa of Iberian pigs. Reproduction **134** 111-121.

Scheepers A., Joost H.G., Schürmann A., (2004) The glucose transporter families SGLT and GLUT: molecular basis of normal and aberrant function. JPEN J Parenter Enteral Nutr **28**(**5**) 364-371.

Schürmann A., Axer H., Scheepers A., Doege H., Joost H. G. (2002) The glucose transport facilitator GLUT8 is predominantly associated with the acrosomal region of mature spermatozoa. Cell Tis Res **307(2)** 237-242.

Shepherd P. R., Gibbs E. M., Wesslau C., Gould G. W., Kahn B. B. (1992) Human small intestine facilitative fructose/glucose transporter (GLUT5) is also present in insulin-responsive tissues and brain. Investigation of biochemical characteristics and translocation. Diabetes **41(10)** 1360-1365.

Shrivastav P., Swann J., Jeremy J. Y., Thompson C., Shaw R. W., Dandona, P. (1989) Sperm function and structure and seminal plasma prostanoid concentrations in men with IDDM. Diabetes Care **12** 742–744.

Senger P L. Pathways to pregnancy and parturition. 2nd Edition. (2003) Current Conceptions Inc, USA.

Spinaci M., Volpe S., Bernardini C., De Ambrogi M., Tamanini C., Seren E., Galeati G. (2005) Immunolocalization of heat shock protein 70 (Hsp70) in boar spermatozoa and its role during fertilization. Mol Reprod Dev **72 (4)** 534-541.

Spinaci M., Volpe S., Bernardini C., de Ambrogi M., Tamanini C., Seren E., Galeati G. (2006) Sperm sorting procedure induces a redistribution of Hsp70 but not Hsp60 and Hsp90 in boar spermatozoa. J Androl **27(6)** 899-907.

Spungin B., Margalit I., Breitbart H. (1995) Sperm exocytosis reconstructed in a cell-free system. Evidence for the involvement of phospholipase C and actin filaments in membrane fusion. J Cell Sci **108** 2525–2535.

Storey B. T. (2008) Mammalian sperm metabolism: oxygen and sugar, friend and foe. Int J Dev Biol **52(5-6**) 427-37.

Suarez S. S. (2008) Control of hyperactivation in sperm. Hum Reprod Update **14(6)** 647-657.

Suarez S. S. and Ho H-C. (2003) Hyperactivated motility in sperm. Reprod Dom Anim **38** 119-124.

Thorens B., Sarkar H. K., Kaback R. H., Lodish H. F. (1988) Cloning and functional expression in bacteria of a novel glucose transporter present in liver, intestine, kidney and beta pancreatic islet cell. Cell **55** 281–290.

Thorens B., Lodish H. F., Brown D. (1990) Differential localization of two glucose transporter isoforms in rat kidney. Am J Physiol **259(6 Pt 1)** C286-294.

Thorens, B. (1992) Molecular and cellular physiology of Glut2, a high-K_m, facilitated diffusion glucose transporter in Molecular biology of receptors and transporters:

bacterial and glucose transporters (M. Friedlander and M. Mueckler, eds) pp. 209-238, Academic Press, San Diego.

Uldry M., Ibberson M., Horisberger J. D., Chatton J. Y., Riederer B. M., Thorens B. (2001) Identification of a mammalian H_-myo-inositol symporter expressed predominantly in the brain. EMBO J **20** 4467–4477.

Uldry M., Ibberson M., Hosokawa M., Thorens B. (2002) GLUT2 is a high affinity glucosamine transporter. FEBS Lett **524** 199–203.

Uldry M., Thorens B. (2004) The SLC2 family of facilitated hexose and polyol transporters. Pflugers Arch - Eur J Physiol **447** 480–489.

Urner F., Leppens-Luisier G., Sakkas D. (2001) Protein tyrosine phosphorylation in sperm during gamete interaction in the mouse: the influence of glucose. Biol Reprod **64** 1350-1357.

Urner F., Sakkas D. (2003) Protein phosphorylation in mammalian spermatozoa. Reproduction **125** 17-26.

Urner F., Sakkas D. (2005) Involvement of the pentose phosphate pathway and redox regulation in fertilization in the mouse. Mol Reprod Dev **70** 494-503.

Vadnais ML, Galantino-Homer HL, Althouse GC. (2007) Current concepts of molecular events during bovine and porcine spermatozoa capacitation. Arch Androl **53(3)** 109-23.

van Gestel R. A., Brewis I. A., Ashton P. R., Helms J. B., Brouwers J. F. Gadella B. M. (2005) Capacitation-dependent concentration of lipid rafts in the apical ridge head area of porcine sperm cells. Mol Hum Reprod **11 (8)** pp. 583–590.

Vera J. C., Rivas C. I., Fischbarg J., Golde D. W. (1993) Mammalian facilitative hexose transporters mediate the transport of dehydroascorbic acid. Nature **364** 79–82.

Visconti P. E. (2009) Understanding the molecular basis of sperm capacitation through kinase design. Proc Natl Acad Sci U S A **106(3)** 667-668.

Watson P.F. (1995) Recents developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev **7** 871-891.

Westhoff D, Kamp G. (1997) Glyceraldehyde 3-phosphate dehydrogenase is bound to the fibrous sheath of mammalian spermatozoa. J Cell Scie **110(15)** 1821-1829.

Wheeler T. J., Hinkle P. C. (1981) Kinetic properties of the reconstituted glucose transporter from human erythrocytes. J Biol Chem **256(17)** 8907-8914.

Widdas, W.F. (1952) Inability of diffusion to account for placental glucose transfer in the sheep and consideration of the kinetics of a possible carrier transfer. J Phys London **118** 23-39.

Williams A. C., Ford W. C. (2004) Functional significance of the pentose phosphate pathway and glutathione reductase in the antioxidant defenses of human sperm. Biol Reprod **71(4)** 1309-1316.

Wood I. S., Trayhurn P. (2003) Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. Br. J. Nutr. **89**(**1**) 3-9.

Wright E. M., Loo D. D., Panayotoya-Heiermann M., et al. (1994) Active sugar transport in eukaryotes. J Exp Biol **196** 197–212.

Wright E. M. Renal Na^+/glucose cotransporters. (2001) Am J Physiol **280** F10–F18.

Wu X., Freeze H. H. (2002) GLUT14, a duplicon of GLUT3, is specifically expressed in testis as alternative splice forms. Genomics **80** 553–557.

Wu X., Li W., Sharma V., Godzik A., Freeze H. H. (2002) Cloning and characterization of glucose transporter 11, a novel sugar transporter that is alternatively spliced in various tissues. Mol Genet Metab **76** 37–45.

Xu, W., Ensrud, K. and Hamilton, D. (1997) The 26 kD protein recognized on rat cauda epididymal sperm by monoclonal antibody 4E9 has internal peptide sequence that is identical to the secreted form of epididymal protein E. Mol Reprod Develop **46** 377–382.

Yanagimachi R. 2008. Mammalian fertilization. In: The physiology of reproduction. Vol. 1 Ed Knobil E., Neil J. D. et al. Raven Press, Ltd. New York.

Yanagimachi R. (2009) Germ cell research: a personal perspective. Biol Reprod **80 (2**) 204-218.

Zhou L., Cryan E. V., d'Andrea M. R., Belkowski S., Conway B. R., Demarest K. T. (2003) Human cardiomyocytes express high level of Na^+/glucose cotransporter 1 (SGLT1). J Cell Biochem **90** 339–346.

Zittle C. A. and Zitin B. (1942) The amount and distribution of cytochrome oxidase in bull spermatozoa. J Biol Chem **144** 99-104.