Review Article

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ISOZYMES OF GLYCOLYTIC PATHWAY IN SPERM: THE UNIQUE SITES FOR CONTRACEPTION

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Abstract: Efficient sperm motility is crucial for reproductive success. Sperm capacitation and sperm activation are prerequisites for sperm-egg interaction and ovum fertilization. Hyperactivity of sperm can be observed if glycolysis is not hampered. How glycolysis, hyperactivity, and sperm fertility are functionally linked is not clear. Glycolysis is the major source of ATP required for sperm flagellar movement, capacitation, and fertilization. The unique isozymic pattern of glycolytic enzymes bound to sperm flagella with specific kinetics seems as a controlling mechanism for requisite energy output of the glycolysis. Furthermore, mammalian sperm contain also atypical isozymes of phosphoglucose isomerase, triosephosphate isomerase, phosphoglycerate mutase, enolase and others. The molecular bases for the testis-specific expression are diverse. For instance PGK-2 is encoded by different gene, while some originate from alternative promoters or by alternative splicing. An unusual feature of GAPDHS is a novel proline-rich N-terminal 105-amino acid sequence not present in other isozymes, whereas ALDOA_V2 and ALDOART1 have a novel 54-55-amino acid sequence at N-terminal region. While LDH-C and PGK-2 lack N-terminal features, Hexokinase, HK1S has an unusual N-terminal spermatogenic cell-specific sequence. This review describes the up-to-date, extended knowledge on isozymic pattern and characteristics of enzymes of pathway of glycolysis in sperm with a view to understand the mechanism of sperm motility and their future potential as targets for contraception by chemical and/or immunological methods.

Keywords: Isozymes in glycolysis; Isozymes of sperm; sperm phosphoglycerate kinase 2; sperm fructose-2, 6-bis phosphatase; glyceraldehyde 3-phosphate dehydrogenase S; aldolase A isozymes; lactate dehydrogenase C

INTRODUCTION

Spermatozoa are highly specialized for delivering the male genome to the egg. Approximately 15–20% of human couples are affected by infertility and about half of these cases of infertility can be attributed to men. In men, the main causes of infertility are oligozoospermia (low sperm production), asthenozoospermia (poor sperm motility), teratozoospermia (abnormal sperm morphology) and azoospermia (lack of sperm production), which account for 20–25% of the infertility cases. Efficient motility is crucial for reproductive success. Sperm capacitation and sperm activation are prerequisite steps before sperm-egg interaction and ovum fertilization. Sperm hyperactivity can be observed if glycolysis is not hampered. Hyperactivity of sperm is important to generate mechanical thrust for its penetration in zona pellucida surrounding the egg. Hyperactivity is apparently induced in the female genital tract when spermatozoa undergo complex metabolic and structural changes that are collectively termed capacitation [Eddy 2007]. How glycolysis, hyperactivity, and sperm fertility are functionally linked is not clear. In other words, we do not know how glycolysis in spermatozoa is organized and regulated. The prime function of sperm metabolism is to provide the flagellar motor with free energy by hydrolysis of ATP, which seems to come mainly through glycolytic pathway (Fig. 1).

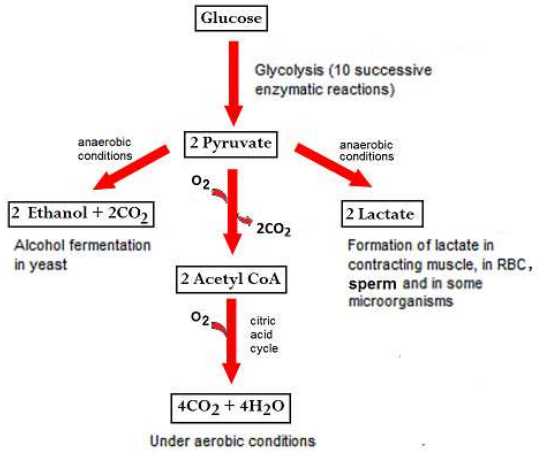

Fig.1. Glycolytic pathway starting with glucose to pyruvate and associated pathways**:** Human spermatozoa rely mainly on glycolysis as the source of ATP fueling the energy-demanding processes of motility and capacitation. Human spermatozoa utilize exogenous pyruvate to enhance glycolytic ATP production and, events that are crucial for male fertility. The glycolysis is dependent on exogenous pyruvate, which indirectly feeds the accelerated glycolysis with NAD^+ through the LDH-mediated conversion of pyruvate to lactate. In addition, oxidative phosphorylation does contribute some ATP for human sperm motility but not enough to sustain high motility (Nascimento *et al.*, 2008; Hereng *et al.*, 2011)

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Spermatozoa are produced in testes, which express variety of isoforms of enzymes associated with energy producing pathways such as glycolysis and pentose shunt pathway. Since, spermatogenesis is highly complex process that involves multiple genes, all of which have not yet been identified, it is important to search for genes that are associated with infertility. Recent studies revealed that genetic abnormalities might affect the function or expression of important proteins and thereby affect male fertility adversely [Gonsalves *et al.,* 2004; Krausz and Giachini, 2007, Nishimune and Tanaka, 2006; O'Flynn *et al.,* 2010; O'Brien 2008].

Earlier studies indicated that some sperm glycolytic enzymes cofractionating with flagellar proteins are associated with insoluble components, and are present in multienzyme complexes [Gillis and Tamblyn, 1984] and difficult to extract. The principal piece region of the sperm flagellum is characterized by the presence of a fibrous sheath (FS). The FS acts as the site for enzymes in the glycolytic pathway that provides energy for the hyperactivation of sperm [Eddy 2007]. In other words FS functions as a scaffold for proteins in signaling pathways that might be involved in regulating sperm maturation, motility, capacitation, hyperactivation, and/or acrosome reaction. Glycolysis is the major source of ATP required for sperm flagellar movement, capacitation, and fertilization [Mukai and Okuno, 2004; Miki *et al.,* 2004]. Studies confirmed that some of the glycolytic enzymes are highly resistant to extraction, including sperm glyceraldehyde 3-phosphate dehydrogenase S (GAPDHS) [Bunch *et al.,* 1998; Westhoff and Kamp, 1997], aldolases (ALDOART1, ALDOA_V2) [Vemuganti *et al.,* 2007], LDHA, and pyruvate kinase (PK) [Feiden *et al.,* 2007, Krisfalusi *et al.,* 2006]. Glycolytic enzymes are known to be products of genes expressed specifically in spermatogenic cells, including lactate dehydrogenase C (LDH-C) [see Gupta 1999], phosphoglycerate kinase 2 (PGK-2) [Boer *et al.,* 1987; McCarrey and Thomas, 1987], GAPDHS [Welch *et al.,* 2006], and two aldolase A isozymes (ALDOART1 and ALDOART2) [Vemuganti *et al.,* 2007]. Others are products of variant transcripts, including spermatogenic cell specific hexokinase type 1 (HK1S) [Mori *et al.,* 1993; Mori *et al.,* 1998] and another aldolase A isozyme (ALDOA_V2) [Vemuganti *et al.,* 2007]. Furthermore, mammalian sperm contain atypical isozymes of phosphoglucose isomerase [Buehr and McLaren, 1984], triosephosphate isomerase [Russell and Kim, 1996], phosphoglycerate mutase [Fundele *et al.,* 1987], and enolase [Edwards and Grootegoed, 1983; Gitlits *et al.,* 2000]. This review describes the up-to-date knowledge on isozymic pattern of enzymes of glycolytic pathway in sperm with a view to identify them as targets for contraception by chemical or immunological methods and to understand infertility in males arising due to changes in their sequential determinants.

ISOZYMES OF GLYCOLYTIC PATHWAY IN SPERM

1. HEXOKINASE:

Hexokinase (HK; EC 2.7.1.1) is the first enzyme in the main pathway of glycolysis, where it phosphorylates glucose to form glucose 6-phosphate (G6P). There are four genes in the hexokinase family in mammals [Wilson, 1995]. Three genes encode isozymes (HK1, HK2 and HK3) of ~100 kDa that contain two homologous sequences (HK domain) in tandem and are inhibited by G6P. The fourth member of this family is glucokinase (GCK), which contains one HK domain and is not inhibited by G6P. HK1 is present in most cell types, whereas HK2 is found predominately in insulin-sensitive tissue. HK3 is present at low levels in liver, lung and spleen, while GCK is present mainly in hepatocytes and pancreatic cells [c/r Nakamura *et al.,* 2008]. Sequence comparisons of HK1 indicate that the N-terminal 15-20 amino acids are 100% conserved. This hydrophobic domain is the putative outer mitochondrial membrane-binding domain of somatic HK1, which is normally cytoplasmic or bound to the outer mitochondrial membrane via contact sites with a voltage dependent anion channel (porin also known as voltage-dependent anion channels; VDACs) through porin biding domain (PBD) [see Nakamura *et al.,* 2008].

1.1. Mouse hexokinase

Mouse spermatogenic cells express three cell-specific variant transcripts from the *Hk1* gene. They were originally named *Hk1-sa*, *Hk1-sb*, and *Hk1-sc* [Mori *et al.,* 1993], but are renamed *Hk1_v1*, *Hk1_v2*, and *Hk1_v3* in accordance with the Mouse Genome Nomenclature Committee. The structural organization of the three *Hk1* variants is shown in Fig. 2. The three variant transcripts encode spermatogenic cell-specific type 1 hexokinase (HK1S). These sequences differ in their 5'untranslated regions, but the open reading frames are alike except for a 69 nucleotide insert in *Hk1_v2* that was referred to as the *Hk1-sb* insert (SBI) [Mori *et al.,* 1993]. A novel feature common to all three variants is the region encoding a 24 amino-acid sequence at the N-terminus that is referred as the spermatogenic cell-specific region (SSR) [Mori *et al.,* 1993; see Adams *et al.,* 1991; Ceser and Wilson, 1998]. Nakamura *et al.,* [2008] examined mRNA from testes of juvenile mice during first synchronous wave of spermatogenesis at the steady-state transcript levels of *Hk1*, *Hk2*, *Hk3*, and *Gck* gene products and showed that levels of their transcripts are quite low in spermatocytes and spermatids while *Gck* transcripts are relatively abundant in spermatids, but that GCK is not detected in spermatozoa. Immunoblotting studies found that HK1S is abundant in sperm and located mainly in the principal piece of the sperm flagellum, where other spermatogenic cell-specific glycolytic

enzymes are also located. Results strongly suggest that HK1, HK2, HK3 and GCK are unlikely to have a role in glycolysis in sperm, but HK1S encoded by *Hk1_v2* and *Hk1_v3* serves this role [Nakamura *et al.,* 2003; 2008]. Unlike LDH-C and PGK-2, HK1S has a unique N-terminal spermatogenic cell-specific region (SSR). HK1S is tethered to muscle-type phosphofructokinase (PFKM) in the principal piece region (Fig.2). The mouse sperm HK1 isoform containing a phosphotyrosine (pY-mHK1) is associated with the plasma membrane fraction of sperm and contains an extracellular domain (Kalab *et al.,* 1994). The germ cell hexokinases might not be localized to the outer mitochondrial membrane and hence could have alternative functions in germ cells and/or sperm. It seems that one of the HK1 isoforms is tyro-phosphorylated during mouse spermatogenesis. [Visconti *et al.,* 1996] (Table-1).

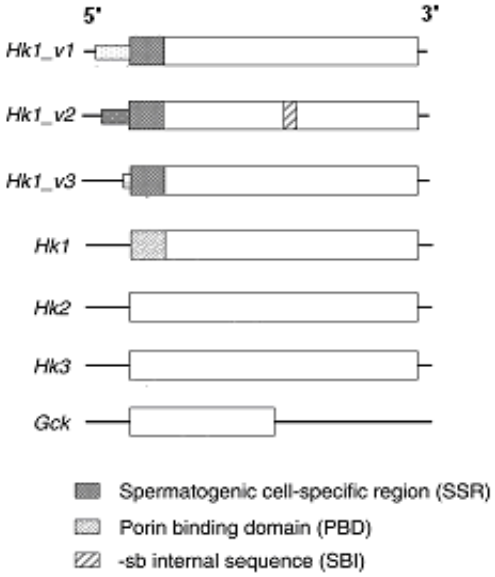

Fig. 2: cDNA structures of hexokinase gene-family: The coding regions of *Hk1* variants and *Hk1, Hk2, and Hk3* are similar in length, whereas the coding region of *Gck* is ~half that of the other members of hexokinase family. The 5′ un-translated regions of *Hk1, Hk1_v1*, *Hk1_v2*, and *Hk1_3* differ in their lengths and sequences (Adapted with permission from Nakamura *et al.,* 2008).

1.2. Human hexokinase

The cDNAs representing three unique human type 1 hexokinase mRNAs (named hHk1-ta, hHk1-tb, and hHk1-tc) are expressed exclusively in testis with a testis-specific sequence not present in somatic Hk1, but lacked the sequence for the PBD. The hHk1-tb and hHk1-tc mRNAs each contained an additional unique sequence, similar to the spermatogenic cell-specific sequence of the mouse mRNAs. RNA from mouse, hamster, guinea pig, rabbit, ram, human, and rat demonstrated absence of PBD in Hk1 from testes of these mammals [Mori *et al.,* 1996]. While Hk1-sa and Hk1-sb are not translated during spermatogenesis, only germ cell-specific Hk1-sc was identified in round spermatids, condensing spermatids, and mature sperm. The sperm Hk1-sc is tyrosine phosphorylated, associated with the mitochondria and the fibrous sheath of the flagellum, and found in discrete clusters in the region of the membranes of the sperm head. The unusual distribution of Hk1-sc in sperm suggests novel functions such as extra-mitochondrial energy production [Visconti *et al.,* 1996; Travis *et al.,* 1998] (Table-1).

Mutation analysis of constructs, (containing a non-hydrophobic, germ cell-specific domain, present at the amino terminus of the Hk1-sc) and targeted to the endoplasmic reticulum and the plasma membrane demonstrated the presence of a complex motif, PKIRPPLTE that represents a novel endoplasmic reticulum targeting motif in Hk1-sc. Constructs based on Hk1-sa demonstrated the specific proteolytic removal of the amino terminal domain, resulting in a protein product identical to Hk1-sc. Such processing might constitute a regulatory mechanism governing the spatial and/or temporal expression of the protein, Hk1-sc [Travis *et al.,* 1999].

2. GLUCOSE 6-PHOSPHATE ISOMERASE OR SPERM ANTIGEN-36:

The glycolytic enzyme, glucose phosphate isomerase (GPI), also known as phosphohexose isomerase catalyzes the interconversion of glucose 6-phosphate and fructose 6-phosphate. GPI is a dimeric enzyme that catalyzes the reversible isomerization of glucose-6-phosphate and fructose-6-phosphate. GPI functions in different capacities inside and outside the cell. In the cytoplasm, the gene product is involved in glycolysis and gluconeogenesis, while outside the cell it functions as a neurotrophic factor for spinal and sensory neurons. The 50-kb gene for human *GPI*, located on the long arm of chromosome 19, has been cloned. Surprisingly, *GPI* mRNA shares high sequence homology to mRNA that codes for neuroleukin (NLK). The NLK is a neurotrophic mediator for spinal and sensory neurons. It is also secreted by activated T lymphocytes and also by tumor cells. The mouse sperm agglutinating monoclonal antibody-36 (mAb A36) was shown to induce extensive, "tangled" sperm agglutination and hence was used to isolate cDNAs encoding its cognate antigen. Sequencing of these cDNAs yielded the complete nucleotide sequence of a 3-kb cDNA that encodes the mAb-related polypeptide, designated sperm antigen-36 (SA-36), composed of 558 deduced amino acids. The SA-36 cDNA displayed 99% homology to GPI/neuroleukin mRNA. It was suggested that mAb A36 cognate sperm surface antigen, encoded by SA-36 cDNA, is a glucose phosphate isomerase/NLK-like protein involved in sperm agglutination [Yakirevich and Naot, 2000]. Buehr and McLaren [1981] had, earlier demonstrated that a unique isoform of glucose phosphate isomerase, absent from somatic tissues,

exists in mouse sperm cells and mouse testes, but only after puberty. Evidently, more studies are needed to elucidate the relationship between sperm isoform of glucose phosphate isomerase /NLK and SA-36 (Table-1).

Table.1: Isozymes of glycolytic pathway in testis/sperm

S. No.	Glycolytic enzyme	Isozyme (s) in testis/sperm	Species	Genes and/or splice variants encoding these isozymes	References
1.	Hexokinase	Hk1-ta, Hk1-tb, and Hk1-tc	Human	*Hk1-ta, Hk1-tb,* and *Hk1-tc*	Mori *et al.*,1996
		HKS1	Mouse	*Hk1_v1*, *Hk1_v2*, and *Hk1_v3*	Mori *et al.*,1993
2.	Glucose 6-phosphate isomerase	Sperm antigen-36	Human	??	Yakirevich and Naot, 2000
3.	Fructose-6-phosphate, 2 kinase / Fructose-2, 6-bis phosphatase	PFKFB or PFK2/FBPase-2	Mouse	*Pfkm_v1; Pfkm_v2, Pfkm_v3, Pfkm_v4*	Nakamura *et al.*,2010;
		tPFK-2,	Rat	*Pfkfb4*	Tominaga *et al.*,1997; Gómez *et al.*,2009;
		pfkfb4	human	*Pfkfb4*	Gómez *et al.*,2005
4.	Aldolase	ALDOART1 ALDOART2	Mouse	*Aldoart1* and *Aldoart2* retrogenes	Vemuganti *et al.*,2007
		ALDOA_V2	Mouse	*Aldoa_v2*, a splice variant of *Aldoa*	Vemuganti *et al.*,2007
5.	Triose phosphate isomerase	TPI	Rat	*Tpi1*	Russel and Kim, 1996
6.	Glyceraldehyde-3 phosphate dehydrogenase	GAPDHS	Human Rat Mouse	*GapdHS* *GapdHS* *GapdHS*	Welch *et al.*,2000 Welch *et al.*,2006 Welch *et al.*,1995
7.	Phosphoglycerate kinase	Pgk2 Pgk2	Human Mouse	*Pgk2* *Pgk2*	McCarrey and Thomas, 1987; McCarrey *et al.*,1992; 1996
8.	Phosphoglycerate mutase	PGAMB PGAM4	Mouse/rat Human	*PGAMB* *PGAM4*	Broceno *et al.*,1995; 1999 Okuda *et al.*,2012
9.	Enolase	enolase S (ENO-S)	Human, Ram and Mouse	? ?	Edwards and Grootegoed, 1983; Force *et al.*,2002
		Eno4	Mouse	*64306537HoRik(Eno4)* and *Gm5506*	Nakamura *et al.*,2009; 2013
10.	Pyruvate kinase	PKS	Boar	*Pkm*	Feiden *et al.*,2007

3. FRUCTOSE-6-PHOSPHATE, 2 KINASE / FRUCTOSE-2, 6-BIS PHOSPHATASE

3.1. Characterization

6-phosphofructokinase (PFK, EC 2.7.1.11) is a main regulatory enzyme of glycolysis. It catalyzes the highly exergonic reaction: fructose 6-phosphate (F6P) + ATP→fructose 1,6-bisphosphate (Fru-1,6P2) + ADP. Several isozymes of Fru-6-P, 2-kinase/Fru-2,6-Pase from mammalian tissues have been characterized. The major isozymes from liver, heart and testis are homodimers, consisting of subunit M_r ranging from 54 to 60 kDa. The catalytic domains of these isozymes are highly conserved, but their N- and C- termini, which are regulatory for enzyme activity, are completely different. In addition to differences in the primary structures, the terminal peptides of the liver and the heart isozymes contain phosphorylation sites and play important roles in regulation of the bifunctional enzyme activity. The effects of phosphorylation suggest the importance of the terminal peptides in the regulation of the enzyme activities, which probably involve the conformational changes induced by interaction of the terminal peptides with the catalytic domain in a specific manner.

On the basis of amino acid homology, the phosphatase activity domain of bifunctional enzyme is structurally related to the phosphoglycerate mutase and acid phosphatase families. The enzyme structure of testis origin shows the location of the C-terminus but does not reveal the N-terminal 36 amino acid residues, suggesting a mobile nature for this terminal tail. To gain insight into the roles of the terminal peptides, 24 and 30 amino acids from the N-terminus of the testis enzyme were deleted. These deletions caused a large increase in $K_{Fru\ 6\text{-}P}$, a decrease in the kinase activity, and an increase in the phsphatase

activity [Tominaga *et al.,* 1993]. To determine the role of phosphorylation, a consensus amino acid sequence (RRAS) was introduced for protein kinase A in the N-terminus of the testis enzyme (which lacks any phosphorylation site). Results suggested that the N-terminus is essential in stabilizing the dimmer structure, probably by strengthening the subunit interaction. Deletion or phosphorylation of the N-terminus weakens the interaction of N-terminus with the catalytic domain, which results in with change of kinetic properties of the enzyme [reviewed in Yuen *et al.,* 1999].

Unlike some other glycolytic enzymes, PFK is not tightly bound to sperm. It was localized in the mid-piece and principal piece of the flagellum as well as in the acrosomal area of the head and in the cytoplasmic droplets released from the mid-piece after ejaculation. In boar spermatozoa, AMP, Fru-2,6-P_2, HC, and citrate act as allosteric effectors controlling PFK activity [Kamp *et al.,* 2007]. The rat testis enzyme, referred to as (RT2K) lacks the regulatory domain, in contrast to the rat liver and the bovine heart enzymes that contain phophorylation site(s) in the N- and the C-termini, respectively. Tominaga *et al.,* [1997] constructed mutant enzymes in which the N- or the C-terminal tail of the testis enzyme was replaced with that of either liver or heart enzyme. The substitution with the N-terminus of the liver enzyme (RLN-RT2K) and c-terminus of heart resulted in a small change in the kinetic properties of Fru 6-P, 2 kinase, but substitution with the N-terminus of heart enzyme increased the $K_{Fru6\text{-}P}$ 18 fold without affecting the V_{max}. The phosphorylation of RLN-RT2K increased $K_{Fru\ 6\text{-}P}$ fivefold as in the liver enzyme but did not affect the Fru 2, 6-Pase, unlike the liver enzyme. Therefore, the N-terminals of the liver enzyme could interact with the kinase domain of the testis enzyme, regulating the kinase activity but was unable to affect the phosphatase domain. These differences could be explained by the large differences in net charges of the terminal tails.

Gómez *et al.,* [2009] analyzed *Pfkfb3* and *Pfkfb4* gene expression in rat testis and testicular cells. During testis development the maximum expression of *Pfkfb3* occurred in pre-puber samples and of *Pfkfb4* in adult samples. The uPFK-2 protein, the product of *Pfkfb3* gene, was present in all the cell types forming the seminiferous epithelium. In contrast, tPFK-2, a product of *Pfkfb4* gene, was restricted to spermatogenic cells. The uPFK-2 in immature sperm was replaced by the testicular isozyme tPFK-2. The tPFK-2 was localized mainly in the acrosomal region of the sperm head and in the mid-piece of the flagellum along with cell-specific glycolytic enzymes.

Nakamura *et al.,* [2010] identified four testis-specific *Pfkm* splice variants, one that overlapped a variant reported previously (*Pfkm_v1*) and three that were novel (*Pfkm_v2*, *Pfkm_v3*, and *Pfkm_v4*). They differ from *Pfkm* transcripts found in somatic cells by encoding a novel 67-amino acid N-terminal extension, the testis-specific region (TSR), producing a spermatogenic cell-specific PFKM variant isozyme (PFKMS). The PFKMS is present in the principal piece and is insoluble in detergents. The TSR was found to interact with glutathione S-transferase μ class 5 (GSTM5), a spermatogenic cell-specific component of the FS. The HK1S is tethered in the principal piece region by PFKMS, which in turn is bound tightly to GSTM5 [Nakamura *et al.,* 2010] (Table-1).

3.2. Allosteric Regulation of PFK

The activity of PFK is allosterically modulated by a number of effectors [see Krause and Wegener 1996]. The control of PFK activity is based on allosteric inhibition by physiological concentrations of its co-substrate ATP, which lowers the affinity for its substrate, so that physiological concentrations of F6P are not sufficient for PFK activity. This inhibition is reinforced by HC (low pH) and by citrate. PFK can be de-inhibited (activated) by positive effectors, some of which are related directly to the turnover of ATP, so that their concentrations are increased whenever ATP turnover is increased. PFK of monkey spermatozoa was shown to be inhibited by ATP and citrate, whereas inorganic phosphate and AMP were activators [Hoskins and Stephens 1969]. Fru-2, 6-P_2 has been demonstrated to activate PFK from rat spermatids and from bull epididymal spermatozoa [Philippe *et al.,* 1986], However, Jones and Connor [2004] did not find any effect of ATP and citrate on PFK activity.

Fructose-2,6-bisphosphate (Fru-2,6-P_2) is a potent allosteric regulator of 6-phosphofructo-2-kinase (Fru-6-P,2-kinase). The levels of Fru-2,6-P_2 are maintained by a family of bifunctional enzymes, 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase (Fru-6-P,2-kinase/Fru-2,6-Pase), which have both kinase and phosphatase activities. Each member of the enzyme family is characterized by their phosphatase:kinase activity ratio (K:B) and their tissue-specific expression. The bifunctional enzyme fructose–6-phosphate, 2 kinase/fructose-2,6-bisphosphatase (Fru-6-P,2-kinase/Fru-2,6-Pase) catalyses the synthesis and degradation of fructose-2,6-bisphosphate and participates in glucose homeostasis by regulating the intracellular concentration of fructose-2,6-bisphosphate (Fru-2,6-P_2). The Fru-2,6-P_2 is both a potent physiological activator of 6-phosphofructokinase, and an *in vitro* inhibitor of fructose-1,6 bisphosphate. The Fru-2,6-P_2 concentration is governed by a tissue–specific nature of bifunctional isozyme, which differs in the relative activities of the

kinase and the phosphatase. The interplay among Fru-2,6-P_2 levels, the enzymes that generate and degrade it, and PFK-1 activity has important consequences for several different aspects of cell metabolism as well as for systemic metabolic conditions. TIGAR (TP53-induced glycolysis and apoptosis regulator), a Fru-2,6 bisphosphatase (Fru-2,6BPase), can also contribute to this complexity and participate in shaping the metabolic profile of the cell [Mor *et al.,* 2011].

The expression of members of *PFKFB* (now *Pfkl*) family in different organs of mice has been shown to respond to hypoxia and the highest hypoxia response was found in liver and testis. Fru-6-P,2-kinase genes, responsive to hypoxia in vivo, indicated a physiological role in the adaptation of the organism to environmental or localized hypoxia/ischemia. The requirement of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases for the regulation of glycolysis in tumor cells and their potential utility as targets for the development of antineoplastic agents has been reviewed by Yalcin *et al.,* [2009].

3.3. Crystal Structure of PFK

The crystal structure of the rat testis Fru 6-P,2 kinase/Fru-2,6-Pase revealed that each enzyme monomer consists of independent kinase and phosphatase domain and the two subunits in the homodimmer are arranged in a head to head manner. The kinase domains are in close contact, forming an extended hydrophobic core between them, while the phosphatase domains are essentially independent of one another. The kinase domain for kinase activity is related to the superfamily of mononucleotide binding proteins with a close relationship to the adenylate kinases and the nucleotide binding portion of the G-proteins. A mutant rat testis isozyme with an alanine replacement for the catalytic histidine (H256A) in the Fru–2, 6-Pase domain retains 17% of the wild type activity [Mizuguchi, *et al.,* 1998]. In the crystal structure of H256A, the Fru-6-P was found at the Fru-2,6-Pase active site, revealing the interaction in substrate/product binding at the active site of the enzyme. A view of the Fru-2, 6-P_2 bound enzyme complex identified the residues responsible for catalysis. The catalytic mechanisms of the wild type and mutant proteins were different. The wild type protein leads to an inefficient transfer of a proton to the leaving group Fru-6-P, which is consistent with a view of this event being rate limiting and responsible for the extremely slow turnover (0.032 s^{-1}) of the Fru-2,6-Pase in all Fru-6P, kinase/Fru-2, 6-Pase isozymes [Yuen *et al.,* 1999].

4. *ALDOLASE*

Aldolase exists as three well-characterized isoforms in vertebrates. Aldolase A is a ubiquitous enzyme, predominantly expressed in muscle, and has been described in the fibrous sheath of mouse and human sperm [Krisfalusi *et al.,* 2006, Kim *et al.,* 2007]. Aldolase B is highly expressed in both kidney and liver whereas aldolase C is found mainly in the nervous system, but has been also identified in bovine spermatozoa [Fiedler *et al.,* 2008]. In addition, three novel aldolase A isoenzymes are expressed in mouse sperm [Vemuganti *et al.,* 2007]. The association of bovine aldolase A with sperm components involved noncovalent, ionic and hydrophobic interactions and did not involve disulfide bonds or covalent bonds. The stability of the bovine aldolase A association with intracellular substructure implies that very specific multiple-ligand bonding is involved. The K_m for fructose-1-phosphate was higher and the activity with fructose-1,6-biphosphate relative to fructose-1-phosphate was much lower than for either liver or muscle aldolase [Gillis and Tamblyn, 1984]. In guinea pig sperm, aldolase A is tightly associated to cytoskeletal structures where it interacts with actin, WAS, and Arp2/3 [Delgado-Buenrostro *et al.,* 2005]. It appears that aldolase A functions as a bridge between filaments of actin and the actin-polymerizing machinery [Chiquete-Felix *et al.,* 2009].

Vemuganti *et al.,* [2007] produced evidence for the presence of three aldolase isozymes in mouse sperm, two encoded by *Aldoart1* and *Aldoart2* retrogenes on different chromosomes and another by *Aldoa_v2*, a splice variant of *Aldoa*. The retrogenes and splice variant have been functional under positive selection for millions of years. Their expression is restricted to the male germline and is tightly regulated at both transcriptional and translational levels. All three isozymes are present only in spermatids and sperm and have distinctive features that may be important for localization in the flagellum and/or altered metabolic regulation. Both ALDOART1 and ALDOA_V2 have unusual N-terminal 54-55-amino acid extensions not found in other aldolases. The novel N-terminal sequences are involved in delivering these enzymes to the principal piece and/or attaching them to the FS [Vemuganti *et al.,* 2007; Bunch *et al.,* 1998; Krisfalusi *et al.,* 2006]. The N-terminal extension of ALDOA_V2 is highly conserved in mammals, suggesting a conserved function in sperm. The N-terminal extensions at FS are required to provide sufficient ATP along the length of the flagellum to support sperm motility [Vemuganti *et al.,* 2007].

5. *TRIOSE PHOSPHATE ISOMERASE*

The triose-phosphate isomerase (TPI) coded by Tpi1 (rodents) or TPI1 (primates/humans) catalyzes the isomerization between glyceraldehyde 3-phosphate and dihydroxyacetone phosphate during glycolysis. Although several studies indicated the presence of TPI in testis and sperm, there is no report on the presence of sperm specific isozyme of TPI. Retinol-induced

cDNAs from rat testis contained a sequence for TPI. The TPI cDNA hybridized with two transcripts in testis. A 1.4-kb transcript, which is the sole transcript found in most tissues, was expressed in the somatic cells of testis, whereas a 1.5-kb transcript was detected only in haploid spermatids. However, only the level of the shorter transcript increased with retinol treatment of testis or of cultured Sertoli cells. Furthermore, screening of an adult rat testis cDNA library with the TPI cDNA yielded a cDNA putatively corresponding to the 1.5-kb transcript. Sequence analyses of the two TPI cDNAs revealed a 100-bp deletion in one cDNA that may be due to use of an alternative polyadenylation signal. These results suggest independent processing mechanisms for TPI expression in the somatic cells and the haploid germ cells of testis [Russel and Kim, 1996]. Octylphenol, an environmental oestrogen down-regulates TPI expression [Li et al., 2011] where as proteome analysis of asthenozoospermic and normozoospermic patients indicated that the proteins falling in group of 'energy metabolism' as TPI were higher in the asthenozoospermic patients. This information has implication in human sperm motility [Siva et al., 2010]. However, further studies are required to establish whether there is a sperm-specific isoform of TPI that plays its role in sperm function [Auer et al., 2004].

6. *GLYCERALDEHYDE-3 PHOSPHATE DEHYDROGENASE*

6.1. Gene organization and functions

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) catalyses the conversion of glyceraldehyde 3-phosphate to D-glycerate 1, 3-bisphosphate in two-coupled steps in glycolytic pathway. The GAPDH in addition to being a classic glycolytic enzyme is a multifunctional protein relevant in cell functions such as endocytosis, DNA replication, DNA excision, DNA repair, translational control of gene expression and apoptosis. GAPDH is also involved in a number of non-glycolytic processes, such as endocytosis, DNA excision repair, and induction of apoptosis. Mammals are known to possess two homologous GAPDH isoenzymes: GAPDH-1, a well-studied protein found in all somatic cells, and GAPDHS, which is expressed solely in testis. In sperm, GAPDHS is restricted to the principal piece of the sperm flagellum where enzyme is tightly associated suggesting its potential role in regulating sperm motility. In mouse testes, a mouse gene *GapdHS* encodes the unique GAPDHS, expressed only in spermatids, which are morphologically differentiated to sperm. Hence the enzyme was called as sperm isozyme, GAPDHS. GAPDHS is highly specific for mammalian sperm and localized along the FS in sperm of boar, bull, rat, stallion and man. GAPDHS supplies energy required for the movement of spermatozoa and is tightly bound to the sperm tail cytoskeleton by the additional N-terminal proline-rich domain absent in GAPDH-1. GAPDH-1 and GAPDHS emerged after duplication during the early evolution of chordates. GAPDHS was subsequently lost by most lineages except lizards, mammals, as well as cartilaginous and bony fishes. In reptilians and mammals, GAPDHS specialized to a testis-specific protein and acquired the novel N-terminal proline-rich domain anchoring the protein in the sperm tail cytoskeleton. Besides testis, GAPDHS of lizards was also found in some regenerating tissues, but it lacks the proline-rich domain due to tissue-specific alternative splicing [Kuravsky *et al.,* 2011]. GAPDHS lacks the sequence similar to the atypical nuclear export signal motif (NES) of the somatic GAPDH-1. The lack of NES correlates with functional peculiarities of the sperm-specific enzyme that is tightly bound to the FS of the sperm flagellum [Kuravsky *et al.,* 2007].

Evidence has been suggesting that sperm specific GAPDHS is covalently linked to the fibrous sheath of axoneme and predominantly present in the circumferential ribs rather than the longitudinal columns [Bunch *et al.,* 1998; Westhoff and Kamp 1997; Tanii *et al.,* 2007]. It is likely that proline rich region of GAPDHS serves as an anchor to FS in sperm. During sperm maturation, most of the cytoplasm of the sperm mid-piece is removed as droplets during the passage through the epididymis [Westhoff and Kamp 1997]. The GAPDHS appears to be the target of several reproductive toxicants that adversely affect male fertility, including α-chlorohydrin, epichlorohydrin, 6-chloro-6-deoxyglucose and ornidazole. These compounds are converted to the common metabolite, 3-chloroacetaldehyde, a competitive substrate inhibitor of the GAPDH isoform in sperm that reduces fertility in several mammalian species.

The mouse *GapdHS* gene encodes a protein of 438 amino acids, whereas the *Gapdh* gene expressed in somatic cells encodes a protein of 333 amino acids. An unusual feature of GAPDHS is a novel proline-rich N-terminal 105-amino acid sequence not present in GAPDH [Welch *et al.,* 2006], The overall homology between GAPDHS and somatic GAPDH is 71% and 49% proline rich. The cysteine required for substrate binding is conserved, as are seven of eight amino acids that form the NAD^+ binding pocket. *In situ* hybridization studies have shown that *GapdHS* mRNA is present only in round and condensing spermatids, first appearing during the cap phase of spermiogenesis [Welch *et al.,* 1995]. Although the multifunctional nature of GAPDH suggests versatility, the mechanisms regulating its expression have been reported by Mezquita *et al.,* [1998]. These workers detected alternative initiation to TATA box and alternative splicing in the 5'-regions of the pre-mRNA, resulting in at least six different types of mRNAs. The amount and the polyadenylation of the GAPDH transcripts increased in mature testis in relation to immature testis and further increased when cell

suspensions from mature testis were exposed to heat shock. Thus alternative initiation, alternative splicing, and polyadenylation could provide the necessary versatility to the regulation of the expression of this multifunctional GAPDHS during spermatogenesis.

The rat GAPDHS has been found to have substantial homology to mouse GAPDHS (94% identity) and human GAPDHS (83% identity) isozymes [Welch *et al.,* 2006]. In sperms GAPDHS exists as the tetrameric molecule bound to FS of the flagellum through the N-terminus of one or two subunits. Comparative study of the amino acid sequences of mammalian GAPDHS revealed conservative cysteine residues (C21, C94, and C150) that are specific for the sperm isoenzyme, GAPDHS and absent in the somatic isoenzyme. Residue C21 can be involved in the formation of the disulfide bond between the N-terminal domain of GAPDHS and other FS proteins [Shchutskaya *et al.,* 2008].

In boar testis, GAPDHS and PKS were first detected in elongating spermatids when both the developing flagellum and the head were labelled with antibodies against GAPDHS and PKS. In contrast, antibodies against somatic GAPDH and PK labelled all developmental stages of germ cells and also neighbouring contractile cells. Thus, the structure-bound sperm-specific enzymes, GAPDHS and PKS, appeared only late in spermatogenesis simultaneously with the development of the structures to which they are bound [Feiden *et al.,* 2008]. Boar spermatozoa contain isoforms of both GAPDH and pyruvate kinase. The sperm-specific GAPDHS and PKS are localized in the principal piece of the boar sperm flagellum as well as in the acrosomal region of the sperm head and tightly bound to cell structures.

Although the steady-state level of *GapdHS* mRNA is maximal at step 9 of mouse spermatogenesis, GAPDHS protein was not detected until steps 12-13 suggesting that *GapdHS* is translationally regulated. Though, a major immunoreactive protein migrating with a molecular weight M_r of 69,200 is observed in condensing spermatids and cauda sperm, additional minor proteins that migrate at M_r 55200, 32500 and 27500 were also detected in sperm. The molecular weight of sperm GAPDHS is higher than the predicted molecular weight of 47445 apparently due to a proline rich 105 amino acid domain at the N-terminus. Recombinant GAPDHS protein lacking the proline rich region migrated at M_r 38250 comparable to somatic GAPDH, which also lacks the proline rich domain. However, the molecular mass of 41.5- and 238-kDa of subunit and native enzyme, respectively suggests that native GAPDHS enzyme is a hexamer.

Expression studies of GAPDHS mRNA in different cancer cell lines show that the content of GAPDHS mRNA is enhanced in some lines of melanoma cells. Three different lines of melanoma cells contain a 37-kDa fragment of GAPDHS polypeptide chain, which corresponds to the enzyme GAPDHS lacking N-terminal amino acid sequence that attaches the enzyme to the cytoskeleton of the sperm flagellum. The GAPDHS is expressed in melanoma cells without N-terminal domain. In melanoma cells both isoenzymes result in the formation of heterotetrameric complexes. It is likely that the expression of GAPDHS in melanoma cells may facilitate glycolysis and prevent the induction of apoptosis [Sevostyanova *et al.,* 2012].

Expression of human GAPDHS in *E.coli* yielded inactive and insoluble protein. Presumably, the N-terminal domain prevents correct folding of the full-length recombinant enzyme. The cytosolic chaperonin TRiC has been shown to assist an ATP-dependent refolding of recombinant forms of GAPDHS, in contrast, to the refolding of muscle isoform GAPDH-1. TRiC is likely to be involved in the refolding of sperm-specific proteins [Naletova *et al.,* 2011]. Compared to muscle GAPDH, $GAPDS_{\Delta N}$ exhibited enhanced thermostability and was much more resistant towards action of guanidine hydrochloride. The enhanced stability of $GAPDS_{\Delta N}$ is likely to be related to some specific features of the GAPDS structure compared to that of the muscle enzyme. It is assumed that high stability of the sperm-specific GAPDS is of importance for the efficiency of fertilization [Elkina *et al.,* 2010]. The GAPDHS activity in sperm with low motility is 2.5-3-fold lower than in samples with high motility. Sperm motility was shown to diminish in the presence of superoxide anion, hydroxyl radical, and hydrogen peroxide. It was suggested that the decrease in the sperm motility in the presence of reactive oxygen species is due to the oxidation of GAPDHS and inhibition of glycolysis [Elkina *et al.,* 2011].

6.2. Crystal Structure

Frayne *et al.,* [2009] obtained soluble recombinant rat sperm GAPDHS as a heterotetramer from the *E. coli* GAPDH in a ratio of 1:3 and solved the structure of the heterotetramer which represents a novel strategy for structure determination of an insoluble protein. A structure was also obtained in presence of glyceraldehyde 3-phosphate which binds at the P(s) pocket in the active site of the sperm enzyme subunit in the presence of NAD. The secondary and tertiary structures of *E. Coli* GAPDS tetramer are highly similar to those of other structurally characterized GAPDHs. Each subunit contains an NAD^+ binding domain and catalytic domain. The NAD^+-binding domain is formed from residues 1–148 and 315–335, including the characteristic Rossmann dinucleotide binding fold, and each binding site is fully occupied by an NAD^+ molecule bound in an extended conformation. The catalytic domain of each subunit is

made up of residues 149–314 and includes the catalytic Cys^{149} at the start of α-helix 4 and the corresponding glyceraldehyde 3-phosphate-binding site (Frayne et al., , 2009). Further insights into the comparison of the structures of human somatic and sperm GAPDHS revealed few differences at the active site and hence did not explain the structural specificity of the sperm isoform for 3-chlorolactaldehyde. However, further analysis of the GAPDHS structure revealed sites that were significantly different from published somatic GAPDH and can be exploited for male contraceptives. Chaikuad *et al.,* [2011] obtained a soluble form of human GAPDHS truncated at the N-terminus ($hGAPDS_{\Delta N}$) in *E. Coli* and crystallized the homotetrameric enzyme in two ligand complexes. The $hGAPDS_{\Delta N}$-NAD^+-phosphate structure maps the two anion-recognition sites within the catalytic pocket that corresponds to the conserved Ps site and the newly recognized Pi site identified in other organisms. The $hGAPDS_{\Delta N}$-NAD^+-glycerol structure shows serendipitous binding of glycerol at the Ps and new Pi sites, demonstrating the propensity of these anion-recognition sites to bind non-physiologically relevant ligands. A comparison of kinetic profiles between $hGAPDS_{\Delta N}$ and its somatic equivalent reveals a 3-fold increase in catalytic efficiency for $hGAPDS_{\Delta N}$. This may be attributable to subtle amino acid substitutions peripheral to the active centre that influences the charge properties and protonation states of catalytic residues [Chaikuad *et al.,* 2011].

7. PHOSPHOGLYCERATE KINASE

7.1. Ontogeny and functions of PGK2

Phosphoglycerate kinase (PGK) (ATP-3-phospho-D-glycerate1-phosphotransferase, EC 2.7.2.3) catalyzes the reversible conversion of 1, 3-diphosphoglycerate to 3-phosphoglycerate, a reaction that generates one molecule of ATP in the glycolytic pathway. There are two monomeric PGK isozymes in eutherian mammals that are encoded by distinct genes. An X-linked phosphoglycerate kinase (*Pgk*) gene is expressed in all mammalian somatic cells and pre-meiotic cells and undergoes dosage compensation. In addition to this X-encoded phosphoglycerate kinase (PGK1 or PGK-A) isozyme, PGK2 (PGK-B) is expressed in some mammalian testis during spermatogenesis and transcribed post-meiotically [Boer *et al.,* 1987; McCarrey and Thomas, 1987; McCarrey *et al.,* 1992]. PGK-2 is encoded by an autosomal retrogene that is expressed only during spermatogenesis. It replaces the ubiquitously expressed PGK1 isozyme following repression of *Pgk1* transcription by meiotic sex chromosome inactivation during meiotic prophase and by postmeiotic sex chromatin during spermiogenesis. Comparison of murine PGK1 and PGK2 isozymes suggested that they evolved from a common ancestral sequence. In contrast to X-linked *Pgk1*, the *Pgk2* gene is located on an autosome in both mouse and kangaroo and has been mapped to the vicinity of MHC gene on chromosome 17 in mouse. However, using sub-cloned fragments of the cDNA, the presence of four independent phosphoglycerate kinase genes was identified. These genes have been mapped to both the human X chromosome (band q13) and chromosome 6 (p12-21.1). The genomic distribution of *Pgk* sequences is conserved in man and mouse, not only for the X-chromosome, but also for linkage to the respective major histocompatibility complexes. Cloning of *X-Pgk* sequences led to the identification of an intronless *Pgk* pseudogene, which is localized proximal to the active gene on the X chromosome [Michelson *et al.,* 1985].

Pgk1 transcription is repressed in spermatocytes due to meiotic sex chromosome inactivation [Turner, 2007; Yoshioka *et al.,* 2007]. Effects of this inactivation are maintained by postmeiotic sex chromatin, and *Pgk1*, like most X-linked genes, remains transcriptionally silent throughout the haploid period of spermatogenesis [Namekawa *et al.,* 2006]. The temporal expression profile of *Pgk2* is opposite that of *Pgk1*, with *Pgk2* mRNA first appearing in preleptotene spermatocytes and increasing in pachytene spermatocytes and spermatids [McCarrey *et al.,* 1992; Yoshioka *et al.,* 2007]. Although *Pgk2* transcripts are present throughout meiotic prophase, PGK2 protein is found only in spermatids, indicating both transcriptional and translational control of expression. Most *Pgk2* mRNA is sequestered in translationally inactive ribonucleoproteins in spermatocytes, while a significant fraction is found on polysomes in round spermatids [Iguchi *et al.,* 2006].

The targeted disruption of *Pgk2* by homologous recombination eliminates PGK activity in sperm and severely impairs male fertility, but does not block spermatogenesis. Mating behaviour, reproductive organ weights and testis/sperm morphology were indistinguishable between $Pgk2^{-/-}$ and wild-type mice. However, sperm motility and ATP levels were markedly reduced in males lacking PGK2. These defects in sperm function were slightly less severe than observed in males lacking GAPDHS, the isozyme that catalyzes the step preceding PGK2 in the sperm glycolytic pathway. Unlike $Gapdhs^{-/-}$ males, the $Pgk2^{-/-}$ males also sired occasional pups. Studies suggest that PGK2 is not required for the completion of spermatogenesis, but is essential for sperm motility and male fertility [Danshina *et al.,* 2010].

7.2. Crystal Structure

PGK2, overexpressed from a plasmid in bacteria, purified and crystallized in three forms: as the apoenzyme, as a complex with 3-phosphoglycerate (3PG), and as a complex with 3PG and ATP. The crystal structures were solved to 2.0 to 2.7 A resolutions (Fig. 3). The overall-fold of PGK2 is nearly identical to

mammalian PGK1 molecules. The apoenzyme is in the "open" form, which means that the N-terminal domain that can bind 3PG and the C-terminal domain that binds ATP are too far apart for the substrates to interact. Binding 3PG causes a 13° rotation that partially closes the structure and causes helix 13, which is disordered in the unliganded structure, to stabilize. Binding ATP leaves the protein in the open configuration but also causes helix 13 to be ordered. Sequence alignment suggests that the active site of PGK2 is essentially identical to that of the cytoplasmic PGK1, but significant differences accumulate on a side of the C-terminal domain away from the active site. These changes may mediate the binding of this isoform to other proteins within the sperm flagellum, while still allowing the hinging action between the domains that is essential to catalytic activity [Sawyer *et al.,* 2008].

Fig. 3. Ribbon diagram of PGK2 structure: The positions of bound 3PG and ATP are shown with red bonds. Helix 13, which becomes ordered by the binding of 3PG or ATP, is shown in yellow. Secondary structural elements discussed in the text are labeled (Adapted with permission from Sawyer *et al.,* 2008).

7.3. Pgk2 gene organization

The nucleotide sequence for the autosomal genomic clone of *Pgk2* showed an open reading frame of 1,251 nt capable of coding a full length PGK protein of 417 amino acids. This coding sequence showed 85% homology with that of the functional human X-linked *Pgk1* gene at the nucleotide level, and 87% homologues at amino acid level with that of the functional *Pgk1* derived enzyme. No introns are present in autosomal sequence, suggesting that this locus may have evolved from a spiced mRNA intermediate by gene processing. The human *Pgk1* gene consists of 11 exons and 10 introns encompassing a region ~ 23-kb in length. The *Pgk2* autosomal sequence is completely deficient in all ten introns and contains characteristics of a processed gene or retroposon including a 165bp remnants of a poly $(A)^+$ tail and bounding direct repeats of which 52% bases are adenines. In the human *Pgk2* gene, direct repeats of seven base pairs are located just 3' to the remnant of the poly $(A)^+$ tail and at 939-945-bp upstream from the start of the coding sequence. Such processed sequences form non-functional pseudogenes that have evolved multiple genetic lesions, which preclude translation of any transcript into a functional polypeptide [McCarrey and Thomas, 1987; McCarrey *et al.,* 1996].

Pgk2 gene is a Transcribed Retroposon of Pgk1: The human *Pgk2* and *ψhPgk1* pseudogenes are retroposons of *Pgk1*. The *Pgk2* retroposon gene arose by reverse transcriptase-mediated processing of a transcript from the *Pgk1* gene. The nucleotide sequence of the *Pgk2* gene suggests that it arose from X-linked *Pgk1* more than 100 million years ago by RNA-mediated gene duplication. Most retroposons form nonfunctional "processed pseudogenes" because they are incomplete copies of their progenitors representing only the transcribed sequences present in the mRNA and deficient in the flanking regulatory (promoter) sequences necessary for direct transcription. For instance, there are at least two pseudogenes of the *Pgk1* gene in the human genome (Michelson *et al.,* 1985) and six pseudogenes of *Pgk1* in the murine genome (Adra *et al.,* 1988). The human *Pgk2* gene was the first functional gene that evolved in this manner (McCarrey and Thomas 1987). Other examples are: *Zfa* and *G6pd2* in the mouse and pyruvate dehydrogenase α-1 (*Pdha1*) in the human [Reviewed in Gupta, 2005]. Zhang and Kleene [1999] suggested that reverse transcriptase cDNA copies of mRNAs are present in meiotic and haploid spermatogenic cells, but these cDNAs are not integrated into genomic DNA. Thus, the *Pgk2* retroposon was formed from an aberrant transcript of *Pgk1* that initiated from an upstream start site, such that the 5' flanking sequence of the *Pgk-1* gene was duplicated along with the coding and untranslated sequence.

Although the *Pgk1* gene is expressed ubiquitously in mammals, expression of the *Pgk2* gene differs widely in different mammalian species, especially when metatherians and eutherians are compared. The expression of PGK2 isozyme is restricted to testicular tissue in the vast majority of eutherians examined. Out of 22 species, only dog and the fox showed some evidence of the PGK2 isozyme in somatic tissues. However, many marsupial species showed at least some *Pgk2* expression in somatic tissues, in addition to a high level expression in the testis. None of the marsupials showed the strict testis specific pattern of *Pgk2* expression seen in the mouse and human [McCarrey, 1994].

Promoter Region of Pkg2: Since the transcriptional regulation of tissue specific gene expression in higher eukaryotes involves multiple levels of control, the Pgk2

gene is no exception. In testis, the transcriptional activity of Pgk2 initiates with the onset of meiosis and continues through all meiotic stages in spermatocytes and in post-meiotic stages in round spermatids. PGK2 transcription ceases in late spermatids when entire genome becomes inactive. Although there are various differences between the *Pgk1* and *Pgk2* promoters, there are certain similarities that form the basis for the hypothesis that the *Pgk2* promoter is derived from the *Pgk1* promoter. These similarities include the absence of a TATA – box sequence in either of the promoter genes and the presence of CAAT/GC box pairs in both the genes. The absence of a TATA box is a common feature of many house-keeping genes, but its absence from tissue specific promoter sequence is rare. The TATA box is typically the key element in the core promoter of tissue specific genes and acts to localize ubiquitous transcription factors, such as the TATA binding protein (TBP) and other associated proteins. These transcription factors in association with RNA polymerase II bind the transcription start point for proper initiation of transcription. (The mechanism underlying the transcription switch from Pgk1 to Pgk2 during spermatogenesis remains obscure.

7.4. Regulation of Pgk2 Gene Transcription

Regulatory Sequences in the 5' Flanking Region: Earlier studies indicated that *Pgk2* expression is regulated at the transcriptional level by core promoter sequences that bind ubiquitous transcription factors and by sequences in a 40-bp upstream enhancer region (E1/E4) that binds tissue specific transcription factors. However, transgenic mice carrying different *Pgk2* promoter sequences linked to CAT reporter gene indicated multiple factor binding regions normally regulating the initiation of transcription from the *Pgk2* promoter, and involvement of multiple transcription factors, which interact themselves to form an enhanceosome like complex with the promoter [Zhang *et al.,* 1999]. Transgenic mice carrying the various upstream regions including the transcription promoter of the testis specific mouse *Pgk2* gene revealed that the 1.4-kb DNA region is sufficient for determining the organ-specific and stage specific transcription of the mouse *Pgk2* gene during spermatogenesis. The 1.4-kb region upstream of the mouse *Pgk2* coding sequence is sufficient for organ, developmental and spermatogenic stage specific transcription of the *Pgk2* gene [Ando *et al.,* 2000]. Although the negative element has been identified but an upstream region longer than 684-bp is needed for transcriptional regulation of the *Pgk2* gene and that negative regulation is necessary for restricting mouse *Pgk-2* transcription in the testis. It is thus possible that transcription of the mouse *Pgk2* gene is regulated in both positive and negative manners.

Sperm, like other metabolically active cells, can activate autosomal *Pgk2* gene to provide necessary PGK in the form of PGK2 during glycolysis. In mouse and human it is transcriptional regulation that dictates expression of the *Pgk2* gene in spermatogenic cells and precludes its expression in somatic cell type. Like other genes, the transcriptional regulation of tissue specific genes, in higher eukaryotes involves multiple levels of control. Transcription of the *Pgk2* gene is controlled by regulatory sequences located in the 5' flanking region of the gene. This region includes both core promoter sequences and tissue-specific enhancer sequences. Core promoter function has been observed in the first 188-bp upstream from the translational start site in the human *Pgk2* gene, including a 70-bp 5'-untranslated region. This core promoter contains a CAAT box and a GC box upstream from the single transcriptional start site, but lacks a TATA box. It has been known that CAAT and GC-boxes act as binding sites for the ubiquitous transcription factors, CTF-1 and Sp-1, respectively [reviewed in Zhang *et al.,* 1999]. Robinson *et al.,* [1989] described a specific region of the *Pgk2* promoter that is required to direct appropriate tissue-specific expression of a reporter gene in transgenic mice. The enhancer activity was located within a 327 base pair region of the 5' flanking region of the *Pgk2* gene, immediately upstream from the core or minimal promoter, which is necessary to direct expression of this gene in spermatogenic cells. Later, tissue specific protein–DNA interactions in this region were also found associated that control *Pgk2* transcription in spermatogenic cells and possibly also with the repression of *Pgk2* transcription in somatic cells. Studies on the appearance and disappearance of DNase I-hypersensitive (DH) sites in *Pgk1* and *Pgk2* each gene and their correlation with transcriptional activity suggested that the occurrence of DH sites is related to periods of active transcription. Results with the *Pgk2* gene indicated that DH sites appear coincident with, or just prior to, transcriptional activation of this gene [Kumari *et al.,* 1996].

Demethylation events in Pgk2 gene: In mammals, DNA methylation occurs at CpG dinucleotides at which methyl groups can become covalently bound to cytosine residues. Many house-keeping genes bear constitutively hypomethylated CpG islands, defined as sequences of 100-bp with relatively high GC content (>50%) that lack the typical under representation of CpG dinucleotides seen in other regions of the mammalian genome. Changes in DNA methylation status have been correlated with many biological processes including gene regulation, X-chromosome inactivation, genomic imprinting, formation of heterochromatin, tumorigenesis, and aging. For many tissue specific genes that don't contain a CpG island, a tissue specific correlation has been demonstrated between hypomethylation and transcription. In the mouse, the 5'-portion of the endogenous *Pgk2* coding sequence undergoes a specific demethylation event

that precedes transcriptional activation by 10-12 days [Ariel *et al.,* 1991; Kafri *et al.,* 1992]. Demethylation denotes the transition of condensed chromatin to an open chromatin configuration in the *Pgk2* promoter required for binding of transcription factors and onset of transcription and that remethylation of the *Pgk2* gene in epididymal spermatozoa is indication of the return to a closed chromatin configuration. McCarrey and colleagues analyzed the methylation pattern of the mouse *Pgk2* gene and have shown that the coding region, as well as the gene flanking region, are highly methylated in *Pgk2* non-expressing cell types, but under methylated in pachytene spermatocytes and spermatids where the *Pgk2* gene is transcribed. Transgenes consisting of the *Pgk2* core promoter ligated to the CAT reporter gene underwent a similar tissue, stage- and cell type specific demethylation in the 5'-portion of the CAT coding sequence, whereas transgenes consisting of the CAT reporter sequence alone, or of the CAT sequence ligated to the CpG island-containing transferrin gene promoter, demonstrated different patterns of demethylation. This indicated that specific promoter sequences can influence the pattern of tissue-specific demethylation within different genes and that a signal for spermatogenic cell specific demethylation resides within the core promoter of the mammalian *Pgk2* gene. These tissue specific protein-DNA interactions and demethylation events distinguish the *Pgk2* gene from the *Pgk1* gene in mice and humans [McCarrey, 1994; Zhang *et al.,* 1998].

8. PHOSPHOGLYCERATE MUTASE

8.1. Muscle specific subunit (M) of phosphoglycerate mutase (PGAM-M)/phosphoglycerate mutase 2 (muscle) Pgam2:

The PGAM proteins function down-stream of PGK in the glycolytic pathway, catalyzing the conversion of 3-phosphoglycerate (3-PGA) to 2-PGA. Phosphoglycerate mutase gene in skeletal muscle *(Pgam-m)* encodes the skeletal muscle specific subunit (M) of the phosphoglycerate mutase (PGAM-M or PGAM2) [Tsujino *et al.,* 1989]. This subunit is also expressed in heart and testis. The *Pgam-m* gene encoding the muscle-specific subunit is transcriptionally activated during spermatogenesis. The muscle-specific *Pgam-m* gene constitutes a meiotic gene and represents a interesting model to study differential tissue specific gene expression. The *Pgam-m* gene is included in the growing list of stage-specific genes co-ordinately expressed during spermatogenesis (Broceno *et al.,* 1995). The PGAM-M type isoform is present in testes from several mammals including humans [Fundele *et al.,* 1987]. The non-muscle-specific, or brain (B) isozyme of human PGAM (PGAM-B) cDNA encodes a deduced protein 254 amino acids long, 79% identical to PGAM-M, and contains a 913-nucleotide 3'-untranslated region, as compared to the unusually short 37-nucleotide 3'-untranslated region of PGAM-M. The cDNAs for the M and B subunits from human and rat have been obtained [Ruiz-Lozano *et al.,* 1994] and the transcription elements involved in muscle specific gene expression of PGAM-M have been documented [Ruiz-Lozano *et al.,* 1994; Broceno *et al.,* 1995]. The mRNA corresponding to the PGAM-M present in testis shows a longer poly (A) tail and begins to be expressed at postnatal day 22, when germ cells start to enter into meiosis. In contrast to what happens during skeletal-muscle differentiation, *Pgam-m* gene expression during spermatogenesis is not coupled to constitutive phosphoglycerate mutase *(Pgam-b)* gene repression.

In comparison to two main extended products of 100 and 102nt detected in skeletal muscle, coincident with the transcription start point previously determined, in testis, two different extended products of 135 and 139nt were mapped upstream of skeletal muscle transcription start point and of the putative TATA box. This suggested a different transcription start point in testis than skeletal muscle for rat *Pgam-m* gene and demonstrated that the transgenic construct contained the endogenous transcription start point for testis and skeletal muscle. The transcription start point for the endogenous *Pgam-m* gene in testis is located upstream from the one in skeletal (25-30nt) muscle (25-30nt) and suggests that *Pgam-m* testis transcription is driven by a TATA less promoter, which is often found when the same gene is selectively transcribed in somatic tissues and during spermatogenesis, although a cryptic TATA box cannot be excluded. A possible clue to the regulatory elements involved in *Pgam-m* testis specific expression may come from the comparison of rat and human *Pgam-m* upstream flanking sequences since the human *Pgam-m* gene is also expressed in adult testis. Sequence alignment between both promoter regions reveals that the homology is essentially reduced to the – 400-bp region of the *Pgam-m* genes (Broceno *et al.,* 1999).

8.2. Phosphoglycerate mutase gene (*PGAM4* also known as *PGAM-B* in mice)

Phosphoglycerate mutase gene (*PGAM-M* or *PGAMB* in mice) is an X-linked retrogene, which is a fundamental gene in human male reproduction and may escape meiotic sex chromosome inactivation. *PGAM4* on the X chromosome might be a functional retrogene expressed in spermatogenic cells that could affect male fertility by producing phosphoglycerate mutase (PGAM)-4 [Disteche *et al.,* 2008; Hendriksen *et al.,* 1997; Khil *et al.,* 2004]. *PGAM4* was first described as a pseudogene transposed from PGAM1, retaining 97.6% of its identity [Dierick *et al.,* 1997]. PGAM4 has high identity of putative amino acid with PGAM1 and contains the LxRHGExxxN motif for PGAM enzymatic activity [Jedrzejas *et al.,* 2000]. The muscle-specific PGAMB subunit was not detected in testes of newborn mice, in which only the PGAM-A isozyme has been observed. In adult males two isozymes are equally

distributed. The PGAMB was first observed between Day 14 and Day 16 of postnatal development and is localized exclusively in germ cells. Among germ cells, PGAMB is detected in pachytene spermatocytes and in spermatids, but not in earlier stages of spermatogenesis. Rat skeletal muscle specific gene for *Pgam-m* is also specifically expressed in meiotic and haploid male germ cells of testis. The 1.3 kb *Pgam-m* promoter contains sufficient sequences to specify temporarily regulated testis-specific expression as well as skeletal muscle expression.

Currently, *PGAM4* is believed to be a functional retrogene because of its neutral theoretical Ka/Ks ratio and the retention of the enzyme active site. Because it is only present in chimpanzees, macaques and humans, *PGAM4* is estimated to have arisen at least 25 million years ago [Betran *et al.,* 2002]. PGAM4 and PGAM1 cDNA sequences differed by 14 bp, but no insertions, deletions, or nonsense mutations within the ORF. In addition, as described [Dierick *et al.,* 1995], *PGAM4* contains several TATA boxes and CAAT boxes upstream from the transcription start site. Moreover, the TGACCTCA sequence at –822 bp is strikingly homologous to the cAMP response element consensus sequence, which has a significant impact on spermatogenesis [Blendy *et al.,* 1996]. Although the PGAM1 promoter sequence contains CpG islands, the promoter sequence of PGAM4 lacks them. This result is intriguing, as PGK1–which contains CpG islands–is expressed ubiquitously, whereas PGK-2 (which does not) is expressed selectively in spermatogenic cells [McCarrey 1990]. PGAM4 is expressed in postmeiotic stages of human spermatogenesis and localized in the principal piece of the flagellum and in the acrosome in ejaculated spermatozoa. In addition, a single nucleotide polymorphism (SNP) causing an amino acid substitution was common in infertile human men and was shown to reduce the enzyme's activity. Results indicate that PGAM4, located on the X chromosome, is a functional retrogene that is associated with male infertility (Okuda *et al.,* 2012).

9. *ENOLASE*

Enolase is a member of a superfamily of enzymes, which are related by their ability to catalyze the abstraction of the α-proton of a carboxylic acid to form an enolic intermediate. Enolase (2-phospho-D-glycerate hydrolase (EC 4.2.1.11) exists as a dimmer in all eukaryotes and catalyzes the conversion of 2-phoshoglycerate in a dehydration step to yield phosphoenolpyruvate in the eighth step of glycolysis that converts 1 molecule of glucose into 2 molecules of pyruvate and 2 molecules of ATP. In higher eukaryotes, enolase exists as three distinct isoforms (α, β, and γ), which are encoded by separate genes. The β isoform appears to be restricted to muscle, while γ enolase has been described as neuron specific (reviewed in Gitlits *et al.,* 2000). In ejaculated spermatozoa, enolase is found as enolase αα (ENO-αα), a ubiquitous form, distributed in most adult cell types, and enolase S (ENO-S), a sperm specific isoform [Edwards and Grootegoed, 1983; Force *et al.,* 2002]. The unusual enolase isoenzyme, ENO-S, is found in human, ram and mouse spermatozoa. This isoenzyme can be distinguished from the somatic enolases by electrophoretic mobility, high thermostability and ability to undergo structural alteration at high temperatures. The pattern of expression of ENO-S during sperm differentiation suggests that this isoenzyme is synthesized relatively late in presence of a haploid genome [Edwards and Grootegoed, 1983]. Both enolase isoforms seem to reflect opposite aspects of sperm cell quality: ENO-αα is associated with abnormal and/or immature spermatozoa where as ENO-S is associated with normal spermatozoa. In testicular sperm, ENO-S was present under 2 main bands named S1 and S3. In epididymal sperm, S1 and S3 bands and a prominent additional S2 band, with the same electrophoretic properties as the S isoform of ejaculated sperm, were visualized. Passage through the epididymis seems to play a major role in the maturation process of ENO-S. The ENO-S/ENO-αα ratio seems to be a valuable marker of the global sperm quality in a given semen sample, and may represent a predictive index of sperm fertilizing potential [Force *et al.,* 2004]. In testes, enolase is also associated with centrosomes. Besides in mature spermatozoa, enolase is also present in residual bodies detached from elongating spermatids with little or no activity in meiotic primary spermatocytes and round spermatids. Nakamura *et al.,* [2009; 2013] found that a pan-enolase antibody, but not antibodies to ENO2 and ENO3, recognized a protein in the principal piece of the mouse sperm flagellum. Database analyses identified two previously uncharacterized enolase family-like candidate genes, *64306537H0Rik* and *Gm5506*. The 64306537H0Rik (renamed *Eno4*) was transcribed in testes. The *Gm5506* gene encodes a protein identical to ENO1 and also is transcribed at a low level in testis. Nakamura *et al.,* [2013] suggested that ENO4 is required for normal assembly of the FS and provides most of the enolase activity in sperm, whereas *Eno1* and/or *Gm5506* may encode a minor portion of enolase activity in sperm.

10. *PYRUVATE KINASE*

Pyruvate kinase (PK, EC 2.7.1.40), the last enzyme of glycolysis, has also been reported to be associated with the acrosome and at the FS of sperm. PK of rabbit spermatozoa could not be solubilised by hypotonic solutions, although its catalytical properties are similar to those of muscle PK (PKM1), which is a soluble protein. Krisfalusi *et al.,* [2006] have reported that PK is bound to the fibrous sheath of mouse spermatozoa where as boar spermatozoa contain a novel pyruvate kinase (PKS) that is tightly bound at the acrosome of

the sperm head and at the fibrous sheath in the principal piece of the flagellum. The midpiece contains a soluble PK. The PKS subunit had a relative molecular mass of 64 ± 1 × 10^3, i.e. slightly higher than that of PKM1, and carried an N-terminal extension (NH_2-TSEAM-COOH) that is lacking in native PKM1. Evidence suggests that PKS is encoded by the *Pkm* gene. Antibodies produced against the N-terminus of PKS (NH_2-TSEAMPKAHMDAG-COOH) did not react with somatic PKs or soluble sperm PK [Feiden *et al.,* 2007].

GLYCOLYTIC ENZYMES AS TARGETS FOR CONTRACEPTION

Glycolysis and sperm motility: Motility is a characteristic function of most of the male gametes and this feature enables spermatozoa to reach a female gamete for fertilization. Several intracellular mediators and exogenous substances have been found to stimulate or inhibit sperm motility. The environmental factors, such as ions and pH, play important role in the activation of sperm motility and metabolism of different species of invertebrates and vertebrates.

Despite extensive research, relatively little is known about how various metabolic pathways operate to induce and sustain motility in mature sperm. A complete understanding of isozymes associated with glycolytic pathway of sperm, enzyme kinetics, energy utilization and the mechanism of motility mediators can ultimately elucidate this complex biological process. Among the two main components of the mature sperm: the head and the flagellum, the head contains the nucleus, acrosome and a small amount of cytoplasm, whereas the flagellum contains the central complex of microtubules forming the axoneme, which is surrounded by outer dense fibers extending from the neck into the principal piece. In order to attain the ability of sperm to fertilize the ovum, sperm undergo several maturational changes during its transit in the epididymal duct where spermatozoa are in a low energy consumption state. However, motility is activated when spermatozoa come in contact with semen components upon ejaculation. Spermatozoa undergo further maturational changes including capacitaion, hyperactivation and acrosome reaction after their deposition in female reproductive tract and before fusion with the female gamete [Hamamah and Gatti, 1998].

Activation of sperm flagellar motility involves activation of both energy metabolism and the motile apparatus. The flagellar movement is generated by the motor activities of the axonemal dynein arms working against stable microtubule doublets. The initiation of the flagellar wave is dependent on the phosphorylation of the axonemal dynein and its activation [Tash, 1989]. The energy released by the hydrolysis of ATP causes the microtubules to slide past one another [Brokaw, 1989; Shingyoji *et al.,* 1977]. Dephosphorylation of dynein by protein phosphatase, calcineurin, reverses the process [Smith, 2002]. Thus the phosphorylation/ dephosphorylation and the corresponding activation and inactivation of the dynein arms occur in an asynchronous manner around the circumference and along the length of the axoneme [Turner *et al.,* 2003; Wargo and Smith, 2003]. Studies suggest that mammalian sperm can produce energy by anaerobic glycolysis, by oxidation of the metabolic products of glycolysis or by oxidation of endogenous substrates. However, there has been a disagreement regarding the relative importance of these three processes because of considerable species variation in metabolic patterns [Miki *et al.,* 2004; Nascimento *et al.,* 2008; Hereng *et al.,* 2011; Odet *et al.,* 2013]. Sperm can use variety of sugars such as glucose, fructose and mannose and can metabolize glycerol, lactate, pyruvate and acetate through glycolytic pathway [Frenkel *et al.,* 1973; Odet *et al.,* 2013]. Mammalian sperm can also produce energy by anaerobic glycolysis or by oxidation of pyruvate. A unique intra-mitochondrial LDH-C4 allows the NADH resulting from pyruvate oxidation to convert pyruvate to lactate [Odet *et al.,* 2013] (Fig. 1). Multiple glycolytic enzymes are localized in the principal piece region of the flagellum in close proximity to the axonemal motor apparatus and often are products of novel spermatogenic cell specific genes [Nakano *et al.,* 2003].

Glycolysis is likely to be utilized in the distal flagella since the enzymes of glycolysis such as hexokinase, GAPDHS and aldolase are localized to fibrous sheath of various mammalian species [Mukai and Okuno, 2004]. Mukai and Okuno [2004] suggested that glycolysis is important for mouse sperm motility, and that respiratory substrates (pyruvate) cannot maintain sperm motility unless glycolysis is also functional. Despite this there has been controversy over the relative importance of glycolysis and mitochondrial oxidation to supply ATP to maintain sperm motility. Miki *et al.,* (2004) suggested that sperm glycolysis is the main pathway to support motility and that the mitochondria were redundant [Ford, 2006]. This was substantiated in knock out (KO) mice of germ cell specific *Gapdhs* which selectively blocks glycolysis. Sperm lacking GAPDHS had defects in sperm motility and fertility with no progressive motility. On the other hand, mice lacking the testis specific cytochrome C, a component of electron transport chain, had the ability to fertilize eggs [Ford, 2006]. Although fertility was significantly reduced in cytochrome C null mice, it was suggested that glycolysis on its own could provide enough ATP to sustain motility and perform sperm function. Indirect evidence for the importance of glycolysis is localization of glycolytic enzymes along the entire length of flagellum to supply ATP where it is

required instead of diffusion from mid-piece mitochondria [Mukai and Okuno, 2004]. *Ldhc* KO mice disrupt male fertility and cause a considerable reduction in sperm glucose consumption, ATP production, and motility. However, in the absence of glucose, sperm from 129S6 KO mice can produce ATP via oxidative phosphorylation, where as in the presence of glucose, oxidative phosphorylation is suppressed and the sperm utilize aerobic glycolysis through Crabtree effect (Odet *et al.,* 2013).

Sperm isozymes as targets for contraception: The important characteristics of sperm specific glycolytic enzymes recommend them as targets for contraceptive development (Gupta 2005, 2012). The restricted expression of novel glycolytic enzymes in the male germline offers the potential for selectively blocking sperm metabolism without inhibiting glycolysis in other tissues. Sperm target isozymes include GAPDHS, PGK2, three mouse sperm aldolases [(ALDOART1 and ALDOART2, aldolaseA splice variant (ALDOA_V2)] and LDH-C in mouse, human and other mammalian sperm. Males lacking GAPDHS are infertile and produce sperm with very low ATP levels, phosphorylation defects and no progressive motility. Both sperm motility and male fertility are also severely impaired in mice lacking PGK-2. These studies demonstrate the importance of glycolysis for sperm energy production. They also provide evidence that inhibition of GAPDHS or PGK-2 should not impair testicular function since sperm production is unaffected in mice lacking either of these isozymes. Studies demonstrate that GAPDHS can be inhibited selectively. These studies underscore the advantage of structure-based drug design to identify selective inhibitors and support the concept that sperm-specific glycolytic enzymes may be excellent contraceptive targets [O'Brien, 2008]**.** The contraceptive activity of α-chlorohydrin and its apparent specificity for the sperm isoform in vivo are likely to be due to differences in metabolism to 3-chlorolactaldehyde in spermatozoa and somatic cells. Further analysis of the sperm GAPDH structure revealed sites in the enzyme that differed from somatic GAPDH structures. Structural and kinetic features of human GAPDHS might provide insightful information towards inhibitor development [O'Brien, 2008]**.**

Chlorinated compounds are known to inhibit glycolysis of spermatozoa in the epididymis. α-chlorohydrin (ACH) is a common contaminant in food. The (S)-isomer, (S)-α-chlorohydrin (SACH) of ACH, is known to cause infertility in animals by inhibiting glycolysis of spermatozoa. In vitro exposure of SACH to isolated rat epididymal sperm indicated that both glycolysis and the cAMP/PKA signaling pathway were impaired by SACH. Results suggested SACH inhibited PTP through blocking cAMP/PKA pathway in sperm, and PTP inhibition may play a role in infertility associated with SACH [Zhang *et al.,* 2012]. The direct inhibition of GAPDH by ornidazole (ORN) suggests that it inhibits without prior conversion outside the cell but inhibition was not stereo-specific. The GAPDH, but not TPI, activity of spermatozoa incubated with ACH and 1-chloro-3-hydroxypropanone (CHOP) was highly correlated with kinematic parameters of spermatozoa incubated in pyruvate- and lactate-free medium. CHOP, unlike ACH was an effective inhibitor of both intact and sonicated cells [Bone *et al.,* 2000]. However, ACH inhibition of GAPDH is not confined to sperm since the sperm enzyme is most sensitive to inhibition [Jelks *et al.,* 2001].

Based on the sperm specific nature of an isozyme of hexokinase and association of a tyrosine-phosphorylated form of Hk1 with sperm, having properties consistent with an integral plasma membrane protein, hexokinase has been suggested as an immunogen for an immuno-contraception [Kalab *et al.,* 1994; Visconti *et al.,* 1996]. Characterization of Hk1 from mutant sperm indicated that at least a portion of the Hk1 present in these cells is an integral membrane protein with an extra-cellular domain located on the sperm head and the flagellum. Anti-phoshotyrosine antibodies demonstrated that Hk1 in mutant sperm is not tyrosine phosphorylated. This suggested a relation between sterility and lack of tyrosine phosphorylation [Olds-Clarke *et al.,* 1996]. Naz *et al.,* [1996], on the other hand, suggested that the hexokinase of 116 kDa present in human sperm is a glycoprotein that is different from the 95-kDa phosphotyrosine protein, and that was not phosphorylated at tyrosine residues. However, its antibodies could cause agglutination and an inhibition of fertilizing ability of human sperm [Naz *et al.,* 1996]. In addition, mouse sperm motility decreased when incubated in media containing glycolytic inhibitor, 2-deoxyglucose (DOG), a glucose analog which inhibits hexokinase at the first step of glycolysis.

The strategy behind sperm-based immuno-contraceptives is to induce antibodies in female reproductive tract against sperm antigen at a sufficient level to block fertilization. It is envisioned that antibodies developed by this vaccine will act before fertilization events, thus terming it as a 'pre-fertilization contraceptive. Advantages of a hypothetical male immune contraceptive include safe, affordable and infrequent administration, long periods of efficacy, and eventual natural reversal. Furthermore, the existing widespread immunization infrastructure could make an immune contraceptive accessible where other methods are not. Disadvantages include a 3-6 week delay to the onset of action and lack of user control over the end of contraceptive effect. Furthermore, a man wouldn't have any direct

indication of when the contraceptive started working, or when it stopped. Constant monitoring of this sort would make immune contraceptives considerably less affordable and convenient. Search of non-reactive antigenic epitopes from LDH-C (Goldberg, 1999) and chemically modified LDH-C4 can offer a potential application for development of a contraceptive vaccine [Gupta and Syal, 1997]. Treatment of female mice with i.p. injection of anti-GAPDHS serum significantly reduced their fertility. Anti-GAPDHS serum caused the agglutination of normal mice sperm in vitro. The anti-GAPDHS antibody was detectable in the sera and uterine fluid of the mice immunized with GAPDHS [Wang *et al.*, 2009]. The role of GAPDHS as an antigen for immuno-contraceptive needs to be investigated in details.

CONCLUSIONS

Studies on isozymes of glycolytic pathway in sperm can be classified into three groups: (i) those which have been reported in details (fructose-6-phosphate,2 kinase/fructose-2, 6-bis phosphatase, glyceraldehyde-3 phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase and LDHC, (ii) those which have started to be investigated (aldolase, hexokinase and enolase) and (iii) those studies which are in very preliminary stage of investigations (glucose 6-phosphate isomerase, triose phosphate isomerase, pyruvate kinase). Present studies with no success for discovery of non-steroidal contraceptive based on inhibition of GAPDHS and LDHC by ACH/gossypol or immunization do not seem promising in the near future [Gupta, 2012].

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ABBREVIATIONS

3PG = 3-phosphoglycerate; Aldoa_v = a splice variant of Aldolase a; ALDOA_V2 = aldolase A variant 2; ALDOA= aldolase A isozyme; ALDOART1= aldolase A retorotranscript 1; ALDOA_V2 = splice variant products of Aldoa; Aldoart1 and Aldoart2 = aldolase A retoro gene transcript 1 and 2; PK = pyruvate kinase; Fru-6-P = fructose-6-phosphate; Fru-1,6P2 = fructose 1,6-bisphosphate; Fru-2,6-P2 = fructose-2,6-bisphosphate; Fru-6-P,2-kinase = 6-phosphofructo-2-kinase; Fru-6P, kinase/Fru-2, 6-Pase isozymes; Fru-2,6BPase = fructose 2,6 diphosphatase; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; GAPDHS = spermatogenic cell specific glyceraldehyde 3-phosphate dehydrogenase; GapdHS= spermatogenic cell specific glyceraldehyde 3-phosphate dehydrogenase gene; GCK = glucokinase; GPI= glucose phosphate isomerase or phosphohexose isomerase; hHk1-ta, hHk1-tb, and hHk1-tc = human testis hexokinase variants; HK1S= spermatogenic cell-specific type 1 hexokinase; Hk1-sa, Hk1-sb and Hk1-sc= germ cell hexokinase a, b and c; Hk1-sa, Hk1-sb, and Hk1-sc or Hk1_v1, Hk1_v2, and Hk1_v3, = germ cell-specific variant transcripts from the Hk1or gene respectively; LDHA = lactate dehydrogenase A; LDH-C= lactate dehydrogenase C; NLK = neuroleukin; PFKM = muscle phosphofructo-1-kinase; PFKMS = Sperm specific PFKM variant isozyme; Pfkm = testis Fru 6-P,2 kinase/Fru-2,6-Pase; Pfkm_v1, Pfkm_v2, Pfkm_v3, and Pfkm_v4 = testis-specific splice variants of Pfkm; PGAM-M = Muscle (M) specific subunit of phosphoglycerate mutase; PGK = phosphoglycerate kinase; PGK1 or PGK-A Phosphoglycerate kinase isozyme-1 or –isozyme A; PGK2 (PGK-B) phosphoglycerate kinase isozyme-2 or –isozyme B; pY-mHk1= phosphotyrosine containing membrane Hex; RT2K= rat testis kinase 2; SA-36 = sperm antigen-36; SSR = spermatogenic cell-specific region; TPI = triose-phosphate isomerase.

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