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Metabolic strategy of boar spermatozoa revealed by a metabolomic characterization

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Abstract Metabolomic characteristics in boar spermatozoa were studied using [1,2-$^{13}C_2$]glucose and mass isotopomer analysis. In boar spermatozoa, glycolysis was the main pathway of glucose utilization producing lactate/pyruvate, whereas no gluconeogenesis was seen. Slight glycogen synthesis through the direct pathway and some incorporation of pyruvate into the Krebs cycle also took place. Neither RNA ribose-5-phosphate nor fatty acid synthesis from glucose occurred despite the detection of pyruvate dehydrogenase activity. In contrast to the known metabolic activities in dog sperm, boar spermatozoa have low levels of energy production and biosynthetic activities suggesting two different metabolic profiles for the two different phenotypes.

Key words: Metabolomics; Mass isotopomer analysis; Glucose metabolism; [1,2-$^{13}C_2$]Glucose; Boar spermatozoa; Glycolysis

1. Introduction

Mammalian sperm is capable of using a wide spectrum of exogenous substrates to maintain its metabolic functions. Among these substrates are monosaccharides, which can be used to provide energy for various cellular functions [1–6]. In some species, these monosaccharides are used with the same efficiency [7]. However, in others, the metabolism of these exogenous substrates may serve specific cell functions. For example, the addition of either fructose or glucose has different effects on functional aspects of dog sperm such as motility [8] or tyrosine phosphorylation [9] patterns. These observations suggest that hexose metabolism plays an important role in energy generation modulating sperm function and survival.

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Abbreviations: GC/MS, gas chromatography/mass spectrometry; G6P, glucose-6-phosphate; PAA, phenylacetic acid; PC, pyruvate carboxylase (EC 6.4.1.1); PDH, pyruvate dehydrogenase (EC 1.2.4.1)

Among mammalian species, sperm may possess a great variety of functional and phenotypic characteristics ranging from the high motility and long life-span of the dog, to the low motility and short survival time of the boar [8,10]. We hypothesize that these functional or phenotypic differences could be linked to changes in sperm energy management, which can be demonstrated by metabolomic and fluxomic profiles. The main aim of this work is to characterize glucose utilization profiles in boar sperm, in order to compare it with what is known for dog sperm, and to correlate the different motility phenotypes with the obtained metabolic profiles. To achieve this objective, metabolomic and fluxomic characterization using [1,2-$^{13}C_2$]glucose and mass isotopomer analysis was performed (see Fig. 1 for further information). The metabolism of glucose via aerobic (Krebs cycle) and anaerobic glycolysis, and the pentose phosphate cycle were studied.

2. Materials and methods

2.1. Materials

[1,2-$^{13}C_2$]D-Glucose (>99% enriched) was purchased from Isotec (Miamisburg, OH, USA), [U-^{14}C]D-glucose and [U-^{14}C]L-lactate were from Amersham (Buckinghamshire, UK), LiCl from Merck (Darmstadt, Germany) and all other reagents from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Cell incubation and metabolite measurement

Boar spermatozoan samples were prepared according to Ballester et al. [11]. Spermatozoan suspension aliquots (sperm population higher than 90%) were incubated without shaking (1 h, 37°C), in the presence of: (i) 10 mM glucose, (ii) 10 mM glucose with 10 mM LiCl, (iii) 10 mM glucose with 10 mM phenylacetic acid (PAA), and (iv) 10 mM lactate. At the end of incubations, cells were obtained by centrifugation at 3000×*g* for 20 s and lactate and glucose were measured in supernatants as previously described [12,13]. In the pellet, glycogen, glucose-6-phosphate (G6P), and protein content were measured as described [11].

To determine substrate oxidation as radioactive CO_2 production, cellular suspension aliquots were incubated for 60 min at 37°C in the presence of either 10 mM [U-^{14}C]glucose (total specific radioactivity: 0.6 μCi/sample) or 10 mM [U-^{14}C]lactate (total specific radioactivity: 0.3 μCi/sample) as in [9].

2.3. Mass isotopomer analysis by gas chromatography/mass spectrometry (GC/MS)

Glucose incubations were repeated using glucose enriched in

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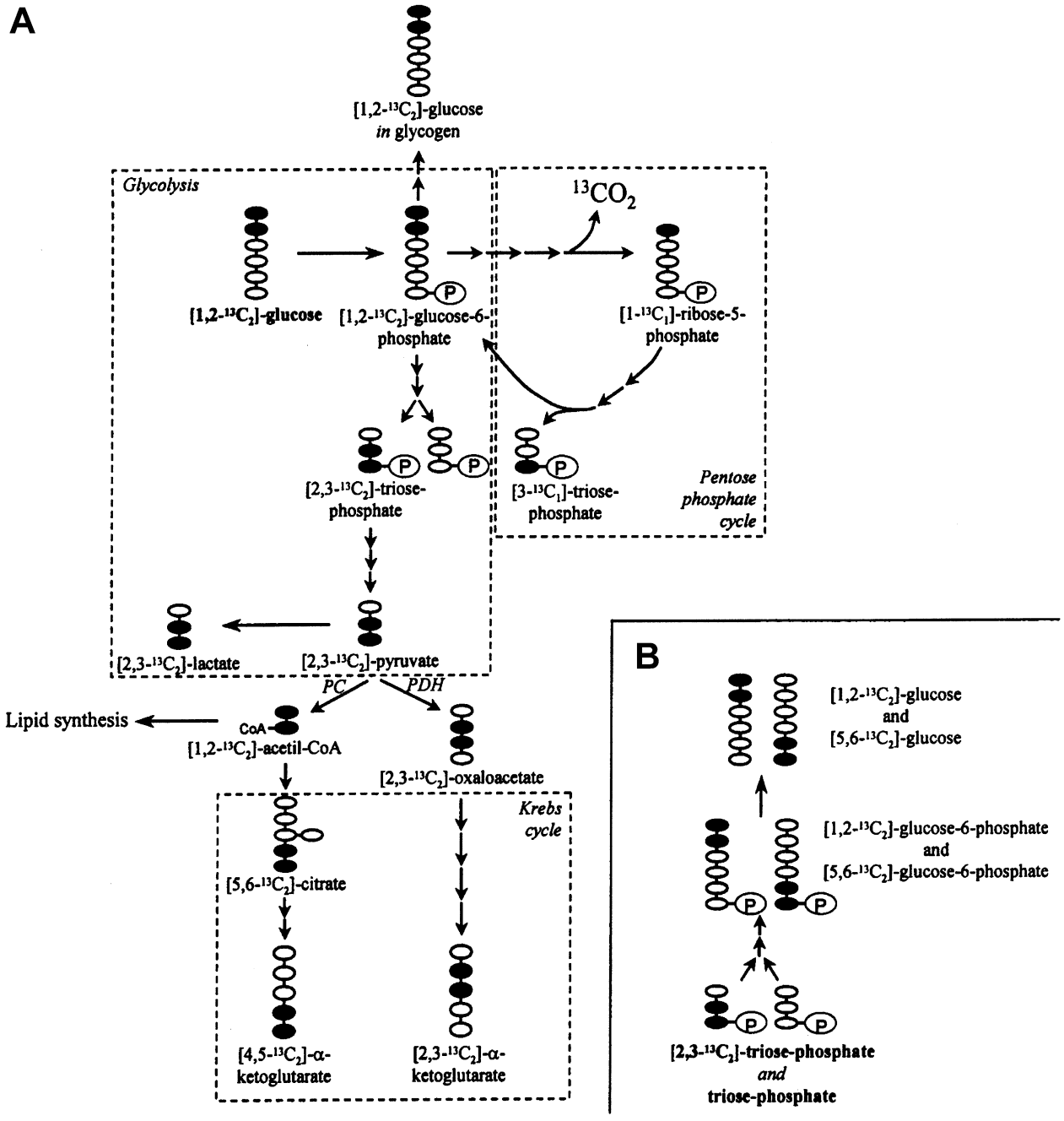

Fig. 1. Expected mass isotopomers in lactate, ribose, newly synthesized fatty acids, and glutamate from incubations with [1,2-$^{13}C_2$]glucose (A) and in newly synthesized glucose from gluconeogenesis (B). When [1,2-$^{13}C_2$]glucose enters into the cell, it is converted into glucose-6-phosphate (G6P), which can undergo glycogen synthesis, and enter the glycolytic pathway or the pentose phosphate cycle (PPC). From glycolysis, two triose-phosphate molecules are formed, one of them with two ^{13}C and the other one without. Both can then form pyruvate, and therefore 50% [2,3-$^{13}C_2$]lactate. Pyruvate can also enter lipid synthesis, forming molecules with a paired number of ^{13}C atoms, or the Krebs cycle, obtaining two different labeling distributions in α-ketoglutarate (which is in equilibrium with medium glutamate) depending on the enzyme used to enter the Krebs cycle: pyruvate carboxylase (PC) or pyruvate dehydrogenase (PDH). When G6P enters the PPC, one ^{13}C is lost in CO_2 formation, giving ribose-5-phosphate with only one labeled atom. This molecule can also enter the non-oxidative pentose phosphate pathway forming triose-phosphate molecules with only one ^{13}C, and all subsequent products labeled in one atom. Furthermore, when products from glycolysis of [1,2-$^{13}C_2$]glucose undergo gluconeogenesis, two different labeling patterns are expected in glucose isotopomers: [1,2-$^{13}C_2$]glucose, which is the initial isotope, and [5,6-$^{13}C_2$]glucose, formed as a result of the isotopic equilibrium between the labeled and unlabeled triose-phosphates.

[1,2-$^{13}C_2$]glucose (48.8%). Mass isotopomers of glucose, glutamate, and lactate from supernatants were determined as in [14–16], and fatty acids and RNA ribose as in [17]. Glucose from glycogen was isolated as previously described [11], and its isotopomers determined as in [14]. Mass isotopomer results are reported as molar fractions of m0, m1, m2, etc., where m0, m1, m2, etc. indicate the number of ^{13}C atoms in each molecule [18].

3. Results and discussion

3.1. Biochemical characterization of boar spermatozoa incubated with glucose

Extracellular lactate concentration, intracellular glycogen, and G6P levels were determined in boar spermatozoa after

Table 1
Biochemical parameters determined in boar spermatozoa incubated with or without 10 mM glucose

	L-Lactate (nmol lactate/mg protein)	Glycogen (nmol glucose/mg protein)	G6P (nmol/mg protein)	CO_2 production (nmol/mg protein)
Krebs $t=1$ h[a]	1.94 ± 1.34	3.72 ± 1.30	0.04 ± 0.02	ND
Glucose 10 mM $t=1$ h	199.3 ± 25.6	3.66 ± 0.90	0.19 ± 0.03	36.3 ± 7.0

Boar spermatozoa were incubated for 1 h at 37°C with or without 10 mM glucose. Extracellular lactate concentration, glycogen, and G6P intracellular levels measured at the end of incubation are provided. CO_2 production after incubation of cells with 10 mM glucose is also indicated. Results are expressed as means ± S.D. from three (lactate, glycogen and G6P) or eight (CO_2) different experiments. ND: not determined.
[a]Lactate, glycogen and G6P at $t=0$ h were not significantly different from after 1 h incubation without glucose.

1 h incubation in Krebs–Ringer solution with or without 10 mM glucose. Further experiments were performed with [U-^{14}C]glucose to measure $^{14}CO_2$ production. Lactate levels at the end of incubation were approximately 2.5 mM, and there was a three-fold increase in G6P concentration, when cells were incubated with glucose (Table 1). No significant changes were observed in glycogen content in both conditions. Finally, a significant accumulation of CO_2 was observed. These results indicate that glucose is mainly converted into G6P by sperm cells before undergoing glycolysis to form lactate and a very small percentage (5.7%) of glucose enters the Krebs cycle.

3.2. Characterization of glycolytic, pentose phosphate, and glycogenesis metabolic pathways in boar spermatozoa

Following 1 h incubation in the presence of 10 mM glucose 48.8% enriched in [1,2-$^{13}C_2$]glucose, the isotopomeric distribution of glucose and lactate was determined using GC/MS. Results show no changes in [1,2-$^{13}C_2$]glucose enrichment at the end of incubation (Table 2), which means that no glucose release was observed. Lactate isotopomer distribution measured at the end of incubation showed that 24.5 ± 0.1% of lactate isotopomers contain two ^{13}C (m2). Since the obtained enrichment equaled the maximum expected enrichment (24.4%), we can infer that all lactate was formed from medium glucose through glycolysis. Moreover, the fact that no m1 isotopomer of lactate was detected suggests that the oxidative pentose phosphate pathway was not significantly active in these cells. The lack of pentose phosphate shunt was confirmed by the lack of ^{13}C label incorporation in ribose-5-phosphate molecules isolated from RNA. Although the pentose phosphate pathway has been demonstrated to play an important role in vital processes in spermatozoa such as capacitation [19], here we demonstrate that this pathway was not operating in ejaculated boar sperm.

At the end of incubation with glucose enriched in [1,2-$^{13}C_2$]-glucose, a very small amount of ^{13}C was incorporated into glycogen glucose as m2 isotopomer (0.55 ± 0.06%). This value represents 0.04% of glucose contribution to glycogen flux with regard to glycolytic flux. Furthermore, the newly incorporated glucose molecules to glycogen came directly from medium glucose as only m2 label was observed. To determine if glycogen synthesis can be stimulated, cells were incubated in the presence of lithium. Lithium is a known activator of glycogen synthesis in the presence of glucose [20]. When 10 mM lithium was added to 10 mM glucose, GC/MS analysis showed that the ^{13}C incorporation as m2 in the presence of lithium rose to 1.07 ± 0.05%. On the other hand, no changes were observed in lactate production, glycogen content or G6P levels.

3.3. Characterization of Krebs cycle and lipid synthesis in boar spermatozoa

The metabolism of glucose at the triose-phosphate level includes reactions involving the Krebs cycle. Lactate formed from triose-phosphate can undergo pyruvate carboxylation initiating gluconeogenesis. To completely determine if the gluconeogenic pathway is active in boar sperm we performed incubation with 10 mM lactate in the medium. Results show no extracellular glucose was produced. Furthermore, G6P fell below the limit of detection, suggesting that sperm were using up this intermediate in the absence of glucose through glycolysis. It also appears that levels of glycogen after incubation with lactate were similar to those observed after incubation with 10 mM glucose (Table 1). Hence, we can conclude that lactate is not used for gluconeogenesis. On the other hand, 10 mM [U-^{14}C]lactate for 1 h induced the production of 13.4 ± 0.1 nmol CO_2/mg protein, indicating that lactate was slightly metabolized through the Krebs cycle probably through pyruvate dehydrogenase (PDH) reactions.

As indicated in Table 1, even though glucose is mainly converted to lactate in boar sperm, a significant amount of CO_2 was also formed. When glucose is oxidized through the Krebs cycle, it is first converted to acetyl-CoA through PDH, which is subsequently incorporated into α-ketoglutarate. Its labeling can be studied by examining glutamate isotopomers of C2–C5 and C2–C4 fragments at the end of incubation (Fig. 2, black bars). Isotopomer m2 of the glutamate C2–C4 fragment can only be formed from [2,3-$^{13}C_2$]α-ketoglutarate because of pyruvate carboxylase (PC). The low value observed is indicative of low PC activity. On the other hand, m2 of the C2–C5 fragment is formed from [2,3-$^{13}C_2$]α-ketoglutarate and [4,5-$^{13}C_2$]α-ketoglutarate because of both PC and PDH activities. Thus, flux through PDH is computed as $m2_{C2-C5}$ minus $m2_{C2-C4}$. Furthermore, [4,5-$^{13}C_2$]α-ketoglutarate contributes

Table 2
Mass isotopomer distribution in medium glucose before and after 1 h incubation

	m0	m1	m2	m3	Σmn
10 mM Glucose, $t=0$ h	0.501 ± 0.003	0.009 ± 0.0002	0.488 ± 0.003	0.002 ± 0.0002	0.991 ± 0.006
10 mM Glucose, $t=1$ h	0.494 ± 0.009	0.010 ± 0.003	0.498 ± 0.007	−0.001 ± 0.002	1.001 ± 0.017

Boar spermatozoa were incubated for 1 h at 37°C with 10 mM glucose enriched in [1,2-$^{13}C_2$]glucose. Glucose isolated from incubation medium before and after incubation of cells was isolated as described in Section 2. Means ± S.D. from three separate experiments are provided. M4, m5 and m6 values are equal to zero. Σmn is the weighted sum of the labeled species (Σmn = m1 × 1+m2 × 2+m3 × 3...).

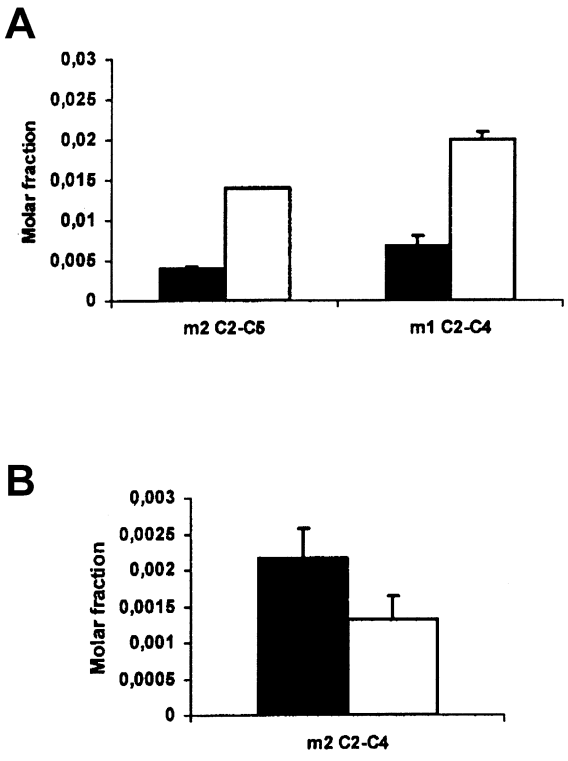

Fig. 2. Mass isotopomer distribution in medium glutamate. Spermatozoa were incubated with 10 mM glucose with (white bars) or without (black bars) 10 mM PAA for 1 h at 37°C. After the incubation glutamate was isolated as described in Section 2. Means ± S.D. of three different incubations are provided for $m2_{C2-C5}$, $m1_{C2-C4}$ (both in A), and $m2_{C2-C4}$ (in B).

to the C2–C4 fragment with only one ^{13}C, resulting in m1 isotopomer. From data in Fig. 2 (black bars), we can see that labeled glutamate is mainly formed through PDH flux.

In order to further demonstrate that the Krebs cycle was indeed active, 10 mM PAA as inhibitor of PC [21] was added to glucose 10 mM. When this pathway is inhibited, pyruvate is forced to enter the Krebs cycle via PDH. When [1,2-$^{13}C_2$]-glucose is used, an increase of the production of [4,5-$^{13}C_2$]α-ketoglutarate is expected. PAA appeared to modify neither lactate synthesis nor glycogen and G6P levels. However, PAA induced a clear redistribution of fluxes in the anaplerotic cycle (Fig. 2, white bars). Thus, when PAA was present, $m2_{C2-C5}$ and $m1_{C2-C4}$ increased 3.5- and 2.8-fold respectively with regard to when PAA was not present, while $m2_{C2-C4}$ decreased 0.4-fold.

Finally, isotopomer analysis in fatty acids showed that no ^{13}C label was incorporated. Thus, even though mass isotopomeric analysis of glutamate has shown PDH was active in boar spermatozoa, the acetyl-CoA formed was mostly utilized for energy production.

4. Conclusions

Two distinctive phenotypes have been observed in spermatozoa of mammalian species. These have been characterized by sperm motility and survival under laboratory and in vivo conditions. These can be represented by (i) the boar sperm phenotype: low average motility and short survival capacity inside the female vaginal tract (about 48 h [22]), and (ii) the dog sperm phenotype: fast average motility [8] and long survival capacity inside the female vaginal tract (about 1 week [10]). This classification, of course, does not preclude the existence of more mammalian sperm phenotypes, since characteristics such as survival capacity and type of movement vary greatly among species.

The obtained results in boar sperm suggest that glucose was subjected to glycolysis, whereas neither gluconeogenesis nor oxidative pentose phosphate pathway were concluded. In addition, slight glycogen synthesis through the direct pathway also occurred. The results also imply that a small percentage of the produced pyruvate was incorporated into the Krebs cycle. However, despite PDH activity, no fatty acid synthesis from glucose took place. The absence of net glycogen synthesis, low G6P levels, and CO_2 production rate of boar sperm contrast with the high G6P levels, glycogen synthesis, and CO_2 production previously reported in dog [9,11,23]. Results obtained here for boar sperm together with previously reported results in dog suggest that different metabolic profiles are associated with these two main types of mammalian sperm cells. In the boar sperm phenotype, the metabolic profile is characterized by a high glycolytic rate with low G6P levels. Boar spermatozoa are capable of utilizing lactate when it is the major carbon source [24]. They lack anabolic metabolism in the ejaculated state. On the other hand, the dog sperm phenotype is characterized by an active anabolic metabolism with high levels of G6P (0.28 nmol/mg protein in 1 h incubation with 10 mM glucose) and efficient glycogen synthesis (200% of synthesis from a basal glycogen of 0.21 μmol glucose/mg protein) and, probably, an active pentose phosphate cycle pathway [9,11,23].

The metabolism of sperm is still poorly understood. The exact type of metabolic profile in a species could be related to the ability of the female genital tract to modulate sperm function. Through evolution, sperm adapt to the nutrient and hormonal environment of the specific secretion of the female genital tract. This mechanism, combined with specific metabolic characteristics of sperm, would establish easy and sensitive mechanisms to optimize fecundation in mammals. The understanding of metabolic profiles of spermatozoa may have great importance in finding treatments for infertility and in improving artificial reproduction methods.

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