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The presence of a high-K_m hexokinase activity in dog, but not in boar, sperm

Josep M. Fernández-Novell[a], Joan Ballester[b], Antonio Medrano[b], Pedro J. Otaegui[c], Teresa Rigau[b], Joan J. Guinovart[a], Joan E. Rodríguez-Gil[b,*]

[a] *Department of Biochemistry and Molecular Biology and IRBB, Barcelona Science Park, University of Barcelona, E-08028 Barcelona, Spain*
[b] *Unit of Animal Reproduction, Department of Animal Medicine and Surgery, School of Veterinary Medicine, Autonomous University of Barcelona, E-08193 Bellaterra, Spain*
[c] *Department of Biochemistry and Molecular Biology, School of Veterinary Medicine, Autonomous University of Barcelona, E-08193 Bellaterra, Spain*

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Abstract **The presence of a high-K_m hexokinase activity was tested in both dog and boar spermatozoa. Hexokinase kinetics from dog extracts showed the presence of a specific activity (dog-sperm glucokinase-like protein, DSGLP), in the range of glucose concentrations of 4–10 mM, whereas boar sperm did not show any DSGLP activity. Furthermore, dog-sperm cells, but not those of boar, showed the presence of a protein which specifically reacted against a rat-liver anti-glucokinase antibody. This protein also had a molecular weight equal to that observed in rat-liver extracts, suggesting a close similarity between both the proteins. This glucokinase-like protein was distributed in the peri- and post-acrosomal zones of the head, and the midpiece and principal piece of tail of dog spermatozoa. These results indicate that dog spermatozoa have functional high-K_m hexokinase activity, which could contribute to a very fine regulation of their hexose metabolism. This strict regulation could ultimately be very important in optimizing dog-sperm function along its life-time.**

Keywords: High-K_m hexokinase; Dog-sperm; Glucokinase-regulatory protein

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1. Introduction

Carbohydrates are, probably, the most important substrates for the maintenance of energy levels in mammalian sperm from fresh ejaculates. Thus, sugars such as glucose, fructose and mannose are utilized as energy sources by sperm cells from species like bull, dog and boar [1]. However, recent findings support the hypothesis that sugars can play another role, at least in some mammalian species, and not only as mere energy substrates. This is especially evident in dog, where the incubation of sperm cells from fresh ejaculates with either glucose or fructose induced hexose-specific changes in functional parameters such as motility [2] or tyrosine phosphorylation patterns [3]. These glucose- or fructose-specific effects were related to specific actions on the majority of the evaluated metabolic parameters, such as intracellular levels of glucose 6-phosphate and glycogen or production of L-lactate and CO_2 [3]. Glucose and fructose also showed separate effects on hexokinase activity [3], and they even induced separate effects, not only on glycogen synthase activity [4], but also on the intracellular, specific location of this enzyme in dog sperm [5]. All of these results indicate that dog sperm would have very sophisticated mechanisms to specifically identify sugars that they are consuming in order to direct them to induce the sugar-linked, functional effects. These mechanisms might be related to the intake of sugars, since the fructose-specific transporter, GLUT5, and the more glucose-specific transporter, GLUT3, are located in separate zones, not only in dog sperm [3], but also in other mammalian species, such as bull, mice and human [6]. However, there are probably other systems that allow sperm to optimize these hexose-differentiating mechanisms, at least in dog.

Vertebrate glucokinase (hexokinase type IV) is a member of the hexokinase protein family which shows some remarkable characteristics that clearly differentiate it from the other mammalian hexokinases. In fact, glucokinase does not have a strict specificity for substrate, since it can phosphorylate not only glucose, but also fructose or mannose [7]. Nevertheless, glucokinase's elevated K_m for glucose, together with its specific expression in the liver and pancreas, allows it to be a sensitive and efficient control step for the maintenance of mammalian glucose metabolism [7]. The existence of a similar, high-K_m hexokinase activity in mammalian sperm could be an efficient system to control that described above, i.e., hexose-specific functional changes observed, at least in dog. Taking this all into consideration, the main aim of this work is to test the presence of a high-K_m hexokinase activity in mammalian sperm, which could act similarly to hepatic glucokinase in the control of sperm's hexose metabolism. For this purpose, sperm cells from dog and boar were used, since they are species which show very different functional characteristics, from their motion parameters (dog cells are fast and linear, whereas boar cells are much slower, see [2,8]) to their life-span after ejaculation (dog spermatozoa last about one week inside the female genital tract, whereas boar cells last only about 48 h, see [9]). In these cells the total hexokinase activity kinetics was deter-

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[*] Corresponding author. Fax: +34-935812006.
E-mail address: juanenrique.rodriguez@uab.es (J.E. Rodríguez-Gil).

Abbreviations: DSGLP, dog-sperm glucokinase-like protein; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TRITC, tetramethylrhodamine isothiocyanate

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mined, and then the presence of proteins with immunological properties like rat-liver glucokinase and glucokinase regulatory protein was tested, in order to find some glucokinase-like, or at least some high-K_m hexokinase, activity in the cells. Our results indicate that dog spermatozoa, but not those of boar, showed a clear high-K_m hexokinase activity, as well as a protein which specifically reacts to an anti-rat-liver glucokinase antibody. This high-K_m hexokinase activity could be related to the dog-sperm's ability to specificially react in front of glucose or fructose.

2. Materials and methods

2.1. Animals and sample collection

Canine semen was obtained from 11 purebred Beagle dogs ranging from 2 to 7 years of age. The care of the dogs followed the guidelines indicated in the Catalan Animal Welfare Law (Generalitat de Catalunya, Spain). Semen was collected once or twice weekly by manual stimulation. Only the sperm-rich fraction of the ejaculates was used.

Porcine semen was obtained from 10 healthy boars ranging from 2 to 5 years of age that belonged to a commercial herd (Servicios Genéticos Porcinos, S.L.; Roda de Ter, Spain). Ejaculates were manually collected and only the rich-sperm fractions were used.

2.2. Total hexokinase activity kinetics

The kinetics of the total hexokinase activity in dog and boar spermatozoa was determined as in [3]. For this, both boar and dog samples were centrifuged at $600 \times g$ for 10 min and were then resuspended in 50 mL of a Krebs–Ringer–Henseleit solution without sugars at 15 °C (KRH− medium; pH 7.4). Sperm were again centrifuged at $600 \times g$ for 10 min and sedimented cells were then resuspended in an additional 50 mL of the KRH− medium. The centrifugation–resuspension step was repeated once more, in order to completely eliminate all substances included in seminal plasma that could affect kinetic and immunological properties of sperm hexokinases. The final, 50-mL-sperm suspension was again centrifuged at $600 \times g$ for 10 min. After this, the supernatant was discarded and the resultant pellets were immediately homogenized by sonication in 250 μL of an ice-cold buffer (pH 7.4) containing 500 mM glycilglycine, 2 M KCl, 100 mM dithiothreitol, 300 IU/mL aprotinin and 100 mM phenylmethylsulfonyl fluoride (hexokinase buffer). Homogenized samples were centrifuged at $10\,000 \times g$ for 15 min at 4 °C and hexokinase activity was measured both in the resultant supernatants and in the pellets. For this purpose, the pellets were washed once in 500 μL of hexokinase buffer and were further resuspended in 250 μL of hexokinase buffer. Hexokinase activity was measured as in [10] with the addition of increasing concentrations of glucose in the reaction buffer after adaptation of the technique to a Cobas Bio autoanalyzer (Roche Biomedical, Basel, Switzerland).

2.3. Immunological techniques

For this experiment, semen samples were pooled both from two (boar semen) or four ejaculates (dog semen). Samples were initially treated through the three times centrifugation/KRH− resuspension washing step described above. After this, both dog and boar sperm cells were suspended in a final 5-mL KRH− medium at 37 °C. Aliquots of the suspension were placed in open vials and incubated with continuous shaking at 37 °C, with the addition to the medium of either glucose or fructose at a final concentration of 10 mM in both cases. Concentrations of sperm cells in the final suspension were of 3.5–4×10^5 spermatozoa/mL in dog samples and $3–6 \times 10^6$ spermatozoa/mL in those of boar. Finally, aliquots were taken at the indicated times and processed for immunological detection.

Western blot analyses were performed in samples homogenized by sonication in ice-cold 10-mM Tris–HCl buffer (pH 7.4) containing 1% (w/v) sodium dodecyl sulfate (SDS) and 1 mM Na_2VO_4 (homogenization buffer, proportion 1:5, v/v). The samples were briefly boiled and were then centrifuged at $10\,000 \times g$ for 14 min at 4 °C. Mammalian sperm has very low amounts of cytoplasm and a very compartmentalized structure [11]. These particularities led us to consider the presence of the tested proteins in either the supernatant or the pellet obtained after homogenization, centrifugation and the boiling of the samples, since proteins could be in a free form or they could be linked to the internal sperm structures. For this purpose, the obtained pellets were resuspended in 20–30 μL of the homogenization buffer, and Western blot analyses were performed in both supernatants and resuspended pellets obtained in all of this process.

Western blot was based on SDS gel electrophoresis [12], followed by transfer to nitrocellulose [13]. The transferred samples were tested with the antibodies at a dilution of 1:1000 (v/v). Immunoreactive proteins were tested using peroxidase-conjugated goat, anti-rabbit second antibody (dilution 1:200, v/v) and the reaction was developed with an ECL-Plus detection system (Amersham, Buckinghamshire, England).

Immunocytochemistry was carried out with spermatozoa seeded onto glass coverslips, which were washed with phosphate-buffered saline (PBS; pH 7.4) and were fixed for 30 min in PBS containing 4% (w/v) paraformaldehyde. The fixed samples were incubated with 1 mg/mL $NaBH_4$ to eliminate autofluorescence, and blocked in 3% (w/v) bovine serum albumin in PBS. Spermatozoa were further incubated with the anti-glucokinase antibody (dilution 1:200, v/v) for 2 h at 15–17 °C, washed with PBS and treated with a tetramethylrhodamine isothiocyanate (TRITC)-conjugated swine anti-rabbit immunoglobulin (Dako, Glostrup, Denmark). Finally, fluorescent images were obtained by a Leica TCS 4D confocal scanning laser microscope (Leica Lasertechnik, Heidelberg, Germany), adapted to an inverted Leitz DMIRBE microscope and a 63× (NA 1.4 oil) Leitz Plan-Apo Lens (Leitz, Stuttgart, Germany). The light source was an argon/krypton laser (75 mW).

2.4. Suppliers

Anti-rat-liver glucokinase and anti-rat-liver glucokinase regulatory protein were produced and tested in the laboratory of Dr. Guinovart (IRBB, Barcelona Science Park, University of Barcelona; see [14]). All of the reactives used were of analytical grade.

3. Results

3.1. Kinetics of dog and boar total hexokinase activity

Supernatants obtained from homogenates of dog sperm showed an increase in total hexokinase activity when it was determined in the presence of glucose in a range from 0.05 mM (1.4 ± 0.1 IU/mg protein) to 2 mM (3.7 ± 0.4 IU/mg protein, see Fig. 1A). A further, and noticeable increase of total hexokinase activity was then determined in a range of glucose concentration from 4 mM (4.2 ± 0.4 IU/mg protein) to 10 mM (8.4 ± 0.8 IU/mg protein, see Fig. 1A). No further increase in total hexokinase activity was detected at glucose concentrations above 10 mM. On the other hand, total hexokinase activity from pellets obtained after homogenization of dog sperm from fresh ejaculates also showed an increase in total hexokinase activity in the glucose concentration range from 0.05 mM (0.60.1 IU/mg protein) to 4 mM (4.0 ± 0.2 IU/mg protein). Again, a further and noticeable increase of total hexokinase activity was observed between 4 mM glucose (4.0 ± 0.2 IU/mg protein) and 10 mM glucose (6.2 ± 0.3 IU/mg protein, see Fig. 1B). These results were reflected in the Lineweaver–Burke representation. Thus, as shown in Fig. 1C, hexokinase kinetics of supernatants from dog-sperm extracts described a biphasic diagram, with two separate lines, the first in the glucose range from 0.05 to 6 mM, and the second in the glucose range from 6 to 50 mM. A theoretical, approximate calculus of the K_m of both lines resulted in values of 8.5 and 0.08 mM, assuming that these values are only approximate. These results were compatible with the presence of a glucokinase-like activity in dog-sperm supernatants. On the other hand, the Lineweaver–Burke representation of pellets from dog-sperm homogenates also showed the presence of two separate lines

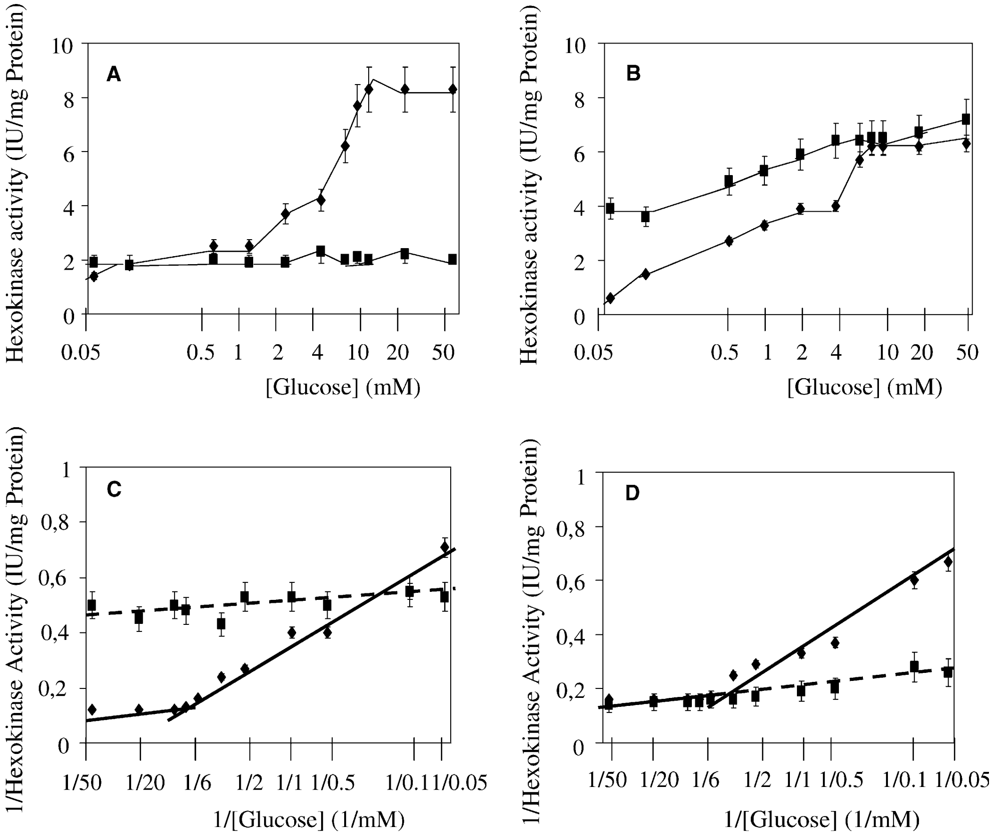

Fig. 1. Kinetics of total hexokinase activity in dog- and boar-sperm extracts. (A and B) Relationship between total hexokinase activity and glucose concentration in supernatants (A) and resuspended pellets (B) from dog (◆) and boar (■) sperm extracts. (C and D) Semi-logarithmic representation between inverse values of hexokinase-specific activity and glucose concentration in supernatants (A) and resuspended pellets (B) from dog (◆) and boar (■) sperm extracts. Lines show the apparent hexokinase activity types from dog (continuous lines) and boar samples (broken lines) revealed by this representation. Results are means ± S.E.M. for eight separate semen samples.

with very different slopes, the first in the glucose range from 0.05 to 6 mM, and the second once again from 6 to 50 mM (Fig. 1D). In this case, the approximate, theoretical values of the K_m of both lines were of 6.9 and 0.09 mM.

Total hexokinase kinetics of boar sperm showed totally different results. Thus, supernatants from homogenates of these cells did not show an appreciable increase, from 0.05 mM glucose (1.9 ± 0.2 IU/mg protein) to 50 mM glucose (2.0 ± 0.3 IU/mg protein, see Fig. 1A), thus indicating the presence of only one hexokinase activity that was very sensitive to the presence of glucose in the medium. Slightly different results were observed in pellets from these homogenates, since in this case there was a progressive increase in hexokinase activity in the glucose range from 0.05 mM (3.9 ± 0.7 IU/mg protein) to 0.5 mM (4.9 ± 0.8 mM, see Fig. 1B), and further increases of hexokinase activity were not noticeable (7.2 ± 0.7 IU/mg protein in the presence of 50 mM glucose). These results were reflected in the Lineweaver–Burke representation, where both supernatants and pellets from boar sperm showed only one line from 0.05 mM glucose to 50 mM glucose (Figs. 1C and D). The calculated, approximate K_m value of this line was of 0.03 mM in supernatants and 0.06 mM in resuspended pellets.

3.2. Presence of an immunoreactive protein against anti-glucokinase antibody

The Western blot using an anti-rat-liver glucokinase antibody revealed the presence of a protein which specifically reacted against this antibody in dog sperm (Fig. 2). This protein was clear in the pellets, with a molecular weight of about 50 kDa (Fig. 2A). On the other hand, the Western blot from supernatants showed two fainter bands of a molecular weight of about 45–50 kDa (Fig. 2B) The intensity and the molecular weight of these bands were not modified after incubation in the

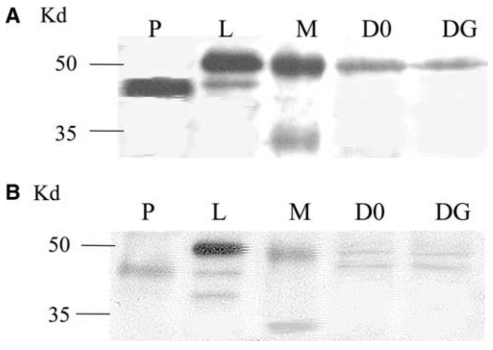

Fig. 2. Western blot against sperm glucokinase. The figure shows two representative Western blots obtained from supernatants (A) and resuspended pellets (B) from dog and boar (P) sperm homogenates. Dog sperm was analyzed from fresh ejaculates (D0) or subjected to incubation with 10 mM glucose for 10 min (DG) before being subjected to Western blot analysis. M: Molecular weight markers. L: Rat-liver extracts. The total number of independent replicates for these Western blots was 5.

presence of 10 mM glucose after up to 30 min (Fig. 2, and data not shown). It is noteworthy that liver extracts used as positive controls showed a positive band for glucokinase of about 50 kDa, which match to those observed in both supernatants and pellets from dog-sperm homogenates (Fig. 2). Finally, boar homogenates did not show any 50-kDa band which could correspond to the result observed in dog extracts, although they revealed a positive signal of about 45 kDa, similar to another non-specific reactivity band detected in liver extracts (Fig. 2).

Confocal images showed the presence of a specific, reactive protein against the anti-rat-liver glucokinase antibody in both the head and the tail of dog spermatozoa from fresh ejaculates (Fig. 3). Tail marking was located at both the midpiece and the main piece, whereas head location was established in both the peri-acrosomal and the post-acrosomal zones. These locations were not significantly modified after incubation in the presence of 10 mM glucose after up to 30 min (Fig. 3C, and data not shown). No positive reaction in front of the anti-rat-liver glucokinase antibody was observed in boar spermatozoa (data not shown).

3.3. Detection of sperm glucokinase regulatory protein

Western blot from dog-sperm extracts did not demonstrate the presence of a specific reactivity against an anti-rat-liver glucokinase regulatory protein antibody, neither in supernatants nor in resuspended pellets obtained after homogenization of samples (Fig. 4, and data not shown). On the other hand, supernatants, but not resuspended pellets, obtained after homogenization of boar spermatozoa showed a clear, specific 70-kDa protein, which was equal to that obtained in rat-liver extracts (Fig. 4A, and data not shown). Molecular weight and density of this band in boar sperm were not modified after incubation with 10 mM glucose and 10 mM fructose after up to 30 min (data not shown).

4. Discussion and conclusions

Our results indicate the presence of a high-K_m hexokinase activity with a similarity to glucokinase in dog sperm, but not in boar cells. This can be sustained by the following facts:

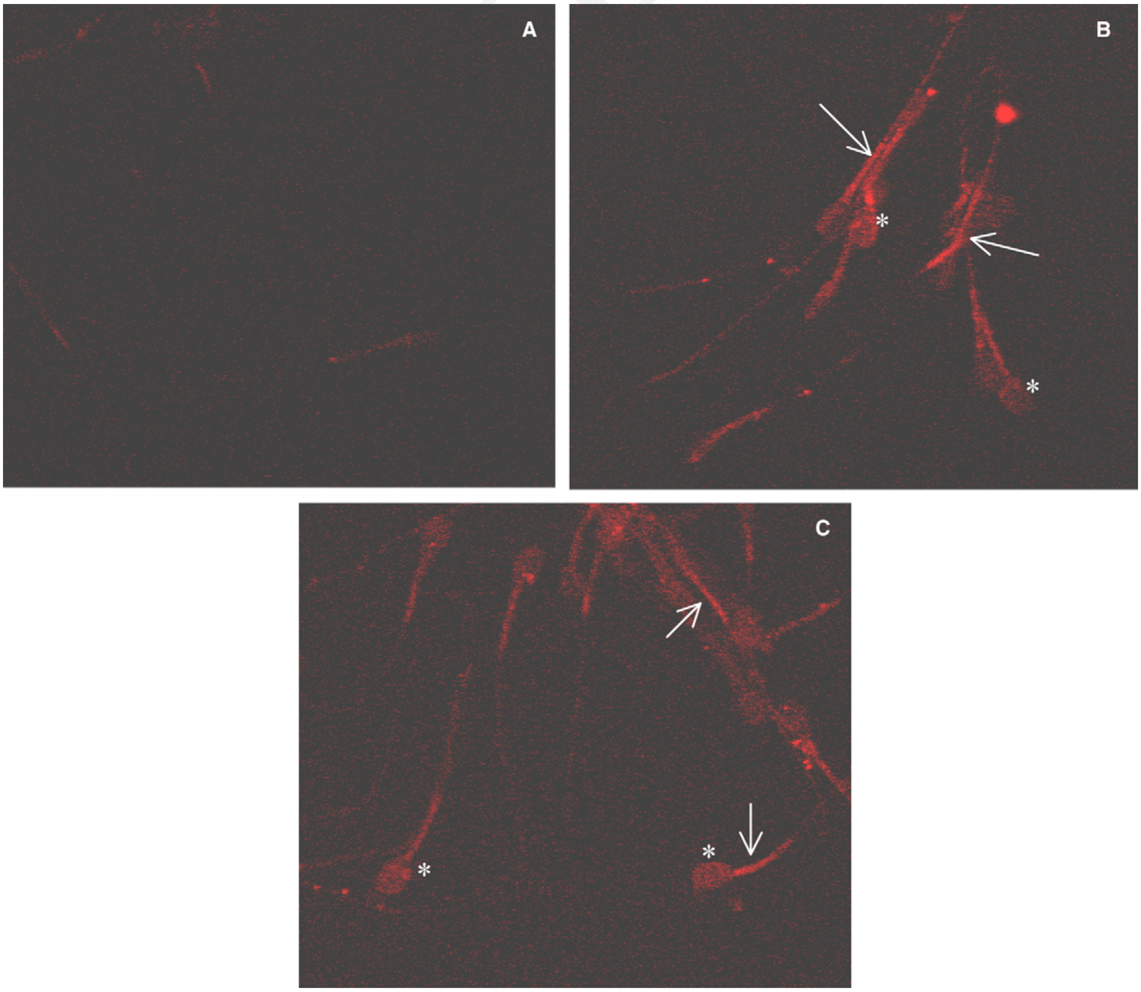

Fig. 3. Immunocytochemistry of dog-sperm glucokinase. The figure shows representative images of dog spermatozoa immunolocalized against glucokinase. A: Negative control. B: Cells from fresh, control samples. C: Cells from samples incubated with 10 mM glucose for 5 min. The total number of independent replicates for these experiments was 5. Images have a multiplication factor of 75. Arrows indicate sperm tails, whereas asterisks indicate sperm heads.

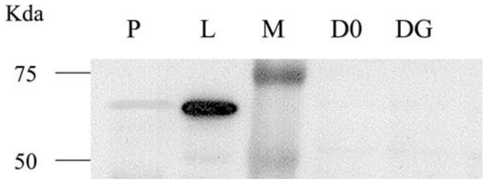

Fig. 4. Western blot against sperm glucokinase regulatory protein. The figure shows a representative Western blot obtained from supernatants obtained after homogenization of either dog or boar (P) sperm homogenates, following the technique described in Section 2. Dog sperm was from fresh ejaculates (D0) or it was subjected to incubation with 10 mM glucose for 10 min (DG) before being subjected to Western blot analysis. M: Molecular weight markers. L: Rat-liver extracts. The total number of independent replicates for these Western blots was 5.

(i) The presence, in dog spermatozoa, of a specific hexokinase activity which shows a high K_m for glucose, which was in the range described for mammalian glucokinase (5–10 mM, see [7,10]).

(ii) The presence of a dog-sperm protein which specifically reacts against an anti-rat-liver glucokinase antibody and, moreover, shows a molecular weight similar to mammalian glucokinase (about 50 kDa, see [7]).

Thus, immunological and kinetic data point towards this interpretation. Of course, we cannot affirm that this protein is truly glucokinase. Moreover, other proteins different from glucokinase have been described with high-K_m characteristics, such as *N*-acetylglucosamine kinase [15] or the high-K_m hexokinase present in the mhAT3F hepatoma cell line (mhAT3F-HK, see [16]). However, kinetic and immunological characteristics indicate that the dog-sperm, high-K_m hexokinase (DSGLP) is more similar to glucokinase than these proteins. Thus, *N*-acetylglucosamine kinase shows a K_m for glucose (370 mM, see [15]) far higher than that of DSGLP (7–9 mM, see Section 3). Furthermore, the comparison of the structural sequences between glucokinase (reference number NP-034422.2, see [17]) and *N*-acetylglucosamine kinase (reference number Q9QZ08, see [17]) revealed a percentage of overall structural affinity of 11.6%. On the other hand, the structural comparison between the epitope utilized to develop the anti-glucokinase antibody [14] and the whole sequence of the *N*-acetylglucosamine kinase rendered the following result:

Epytope of liver glucokinase:

414-**K**LHPSF**KE**R**F**ASVR-428

Sequence of *N*-acetylglucosamine kinase which rendered the higher homology:

274-**K**SWELL**KE**G**F**LLALT-288

As shown in this comparison, in the best case only four aminoacids, which were not linear (highlighted letters), are the same in both sequences. These data seem to indicate that our antibody has a low degree of affinity for *N*-acetylglucosamine kinase. Furthermore, the theoretical molecular weight of *N*-acetylglucosamine kinase, calculated from its aminoacidic composition [17], is about 37–38 kDa. Our Western blot analysis did not detect any band with could be included in the 35–40 kDa molecular-weight range. Summing up all of these data, we can conclude that *N*-acetylglucosamine kinase does not seem to be DSGLP. On the other hand, mhAT3F-HK also shows a K_m for glucose (40 mM, see [16]) higher than DSGLP. Moreover, although this protein recognized an anti-glucokinase antibody, its molecular weight was about 30 kDa [16]. Our results did not reveal the presence of any protein with this molecular weight. The sum of all of these results, of course, does not preclude the presence of *N*-acetylglucosamine kinase and mhAT3F-HK in dog sperm. However, our results also indicate that DSGLP with immunological properties similar to rat-liver glucokinase, regardless of the existence of other high-K_m hexokinases.

Nevertheless, some differences between glucokinase and DSGLP exist. Thus, glucokinase activity is mainly regulated in two ways, first through controlling its expression [7]. However, mammalian sperm does not have the ability to express its DNA to synthesize proteins [11], so, in this manner, dog sperm cannot control DSGLP activity through this way. The second way is through changes in its spatial position inside the cell [14]. This latter control is achieved through a glucose-modulated linking between glucokinase and the glucokinase-regulatory protein, which displaces glucokinase in the hepatocyte to areas with or without glucose, thus controlling its ability to phosphorylate the monosaccharide [14]. On the other hand, displacements of glucokinase-regulatory protein through the hepatocyte to achieve its control of glucokinase activity are possible due to the hepatocyte being a cell with an active and more-or-less relaxed nuclear structure as well as with a great amount of cytoplasm. Spermatozoa have neither requirement, since their amount of cytoplasm is very scarce and their nuclear structure is condensed and very inactive, totally different from that observed in hepatocytes [18]. Moreover, the presence of a clear immunoreactivity of glucokinase-regulatory protein in boar sperm, which did not show DSGLP activity, suggests the lack of a clear regulatory role for the glucokinase-regulatory protein in dog sperm. Thus, DSGLP activity regulation would have probably been achieved by other ways, like phospho-dephosphorylation mechanisms that are also operative on glucokinase [19].

The functional role of DSGLP could be explained as a regulatory mechanism of dog-sperm hexose metabolism. Previous reports have shown that dog-sperm cells have a very complex hexose metabolism, which includes the presence of anabolic paths like glycogen metabolism, and differentiated, functional roles for separate hexoses [2,4,5]. The elevated K_m for glucose shown by DSGLP could be an efficient system to control glucose-specific mechanisms of regulation of dog-sperm functionality, in a similar way that glucokinase controls glucose-induced changes in hepatocytes and pancreatic β-cells [7,10].

It is noteworthy that DSGLP is present in dog spermatozoa, but not in boar cells. This would mark great differences in hexose metabolism and, hence, in the energy status management of both species. In fact, hexose metabolism of spermatozoa from several mammals like boar or bull are basically glycolytic, with elevated L-lactate formation rates, low glucose 6-phosphate levels, low activity in anabolic pathways like glycogen synthesis and small differences, if any, in the selective utilization of hexoses such as glucose or fructose as energy substrates [20,21]. In these spermatozoa, the presence of DSGLP makes no sense, since they utilize different monosaccharides in the same way for the same ultimate necessity, the attainment of energy. Nevertheless, as commented above, energy management of dog spermatozoa is very different, and the selective, functional utilization that these cells have of glucose and fructose can easily explain the necessity of DSGLP. Thus, our results indicate that there is not a single, simple mechanism that explains the regulation of mammalian sperm function under the energetic point of view. This has to be taken into consideration when trying to apply new strategies in the conservation of sperm, since it would vary depending on the specific metabolic and hexose-related functional profiles of each species.

5. Conclusions

The presence of DSGLP in dog spermatozoa would play an important role in the control of both the energy management pathways and the hexose-related functional mechanisms that dog spermatozoa show from fresh ejaculates. Notwithstanding, at this moment we have no real knowledge of the exact physiological role and control mechanisms of DSGLP, and experiments regarding substrate specificity, besides purification, sequencing and cloning of DSGLP, will be needed to clarify these important points.

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