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Research

Differential effects of glucose and fructose on hexose metabolism in dog spermatozoa

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Incubation of dog spermatozoa with 10 mmol l^{-1} glucose or fructose rapidly increased the intracellular content of glucose 6-phosphate and fructose 6-phosphate, although the effect of fructose was greater. These effects were correlated with increases in ATP, ribose 5-phosphate and glycogen contents, and in the rates of formation of L-lactate and CO_2. In all cases, except for ATP and glycogen, the effect of fructose was greater than that of glucose. The total hexokinase activity of the crude extracts of dog spermatozoa was more sensitive to fructose than to glucose at lower concentrations (0.1–3.0 mmol l^{-1}). Both monosaccharides induced a fast and intense increase in the overall tyrosine phosphorylation of dog spermatozoa, although their specific induced-phosphorylation patterns differed slightly. Glut 3 and Glut 5 hexose transporters were the main hexose transporters in dog spermatozoa; however, other possible SGLT family-related hexose transporters were also localized. These data indicate that, at concentrations from 1 mmol l^{-1} to 10 mmol l^{-1}, fructose has a stronger effect than glucose on hexose metabolism of dog spermatozoa. These differences appear to be related to variations in the sensitivity of hexokinase activity. Moreover, the differential hexose metabolism induced by the two sugars had distinct effects on the function of dog spermatozoa, as revealed by the diverse patterns of tyrosine phosphorylation.

Introduction

Fresh mammalian spermatozoa obtain the energy necessary to maintain motility by introducing appropriate substrates into the glycolytic pathway and the Krebs cycle. It is generally accepted that monosaccharides provide the external source of substrates with which the energy status of these cells is maintained. Thus, mature mammalian spermatozoa have the ability to form lactate from several hexoses, such as glucose, fructose and mannose (Mann, 1975). Moreover, these spermatozoa can use three-carbon molecules, such as glycerol, by introducing them into the glycolytic pathway (Jones *et al.*, 1992). However, species differ greatly in their ability to use sugars throughout the glycolytic pathway (Rikmenspoel and Caputo, 1966; Hammersted and Lardy, 1983; Jones and Connor, 2000). It is necessary to study the maintenance and regulation of the energy status of spermatozoa to understand their survival and the way by which they modulate their activity during their life cycle. Nevertheless, few studies have addressed this question.

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Glucose and fructose are two of the most commonly found monosaccharides in mammalian seminal plasma, although other sugars, such as sorbitol or mannose, can also be detected (Setchell and Brooks, 1988). The presence of either glucose or fructose can affect the function of mammalian spermatozoa in several ways. Glucose concentrations of about 5 mmol l^{-1} produce much higher penetration rates than do fructose or mannose in human spermatozoa (Rogers and Perreault, 1990). Moreover, glucose, but not fructose, produces a high fertility rate and capacitation-like changes in the chlortetracycline fluorescence pattern of mouse spermatozoa subjected to '*in vitro*' capacitation (Fraser and Herod, 1990). This finding indicates that the changes in the sperm membrane, which are needed for penetration, are glucose-dependent (Fraser and Herod, 1990). Despite these data, the mechanisms by which glucose, but not fructose, induces these effects are not known, although they are probably related to distinct effects in the management of sperm function. There are also no reports about the role of glucose or fructose in the physiology of dog spermatozoa. In this respect, the motility patterns of dog spermatozoa incubated with either fructose or glucose differ (Rigau *et al.*, 2001), thereby indicating that these sugars act differently on the mechanisms controlling motility. Dog spermatozoa provide a good model in which

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to study the effects of monosaccharides, as canine seminal plasma does not contain significant amounts of glucose, fructose, sorbitol or mannose (Rigau *et al.*, 2001). Thus, freshly ejaculated dog spermatozoa have no contact with these sugars before processing. However, the precise mechanisms by which these two sugars induce these separate effects remain unclear.

The aim of this study was to determine the effects of glucose and fructose on hexose metabolism of mature dog spermatozoa and, in this way, to identify the mechanisms used by these monosaccharides to induce their differential functional effects. For this purpose, the intracellular content of some of the main markers of hexose metabolism of spermatozoa, such as glucose 6-phosphate, ribose 5-phosphate, glycogen and ATP, and also the rates of formation of L-lactate and CO_2, were determined. The tyrosine phosphorylation pattern of dog spermatozoa was also studied to determine whether these sugars could modify it. The study was completed with the determination of the total hexokinase activity of sperm extracts and the main membrane hexose transporters present in eukaryotic cells.

Materials and Methods

Materials

Rat anti-Glut 1, anti-Glut 2, anti-Glut 3, anti-Glut 5 and anti-SGLT-1 were from Chemicon International (Temecula, CA). Anti-mouse phosphotyrosine (PY20) was from Transduction Laboratories (Lexington, KY). [U^{14}C]glucose and [U^{14}C]fructose were purchased from Amersham (Little Chalfont). Glucose and fructose were obtained from Merck (Darmstad). All other reagents were of analytical grade and were supplied by Sigma (St Louis, MO), Merck, BioRad (Hercules, CA) and EMS (Fort Washington, PA).

Animals and sample collection

Semen was obtained from 11 purebred Beagle dogs aged 2–6 years. The dogs were kept in outdoor kennels, exercised twice a day and fed a balanced diet with free access to water. Semen was collected once or twice a week, without using a female, by manual stimulation into warmed (37°C) sterile glass or plastic funnels. Only the sperm-rich fraction of each ejaculate was used.

Processing of semen samples

The freshly obtained sperm-rich fraction was centrifuged immediately at 600 ***g*** for 10 min and the seminal pellet obtained for four ejaculates was pooled and resuspended immediately after centrifugation in a Krebs–Ringer–Henseleit medium (pH 7.4) at 37°C without any monosaccharide. The sperm suspension was centrifuged again at 500 ***g*** for 10 min and resuspended in the same medium. Finally, spermatozoa were resuspended in 5 ml Krebs–Ringer–Henseleit medium (pH 7.4) at 37°C without monosaccharides. Aliquots of the suspension were placed in open vials and incubated with continuous shaking at 37°C with additions of fructose or glucose to the medium. The concentrations of sperm cells in the final suspension ranged from 3.5×10^5 to 4.0×10^5 spermatozoa μl^{-1}, when evaluated in either a Neubauer or Thoma haemocytometer cell chamber. Finally, aliquots were taken at the times indicated and were centrifuged at 1000 ***g*** for 1 min, and supernatants and pellets were frozen immediately in liquid nitrogen. These samples were stored at –80°C until analysis. In all cases, a separate 10 µl aliquot was collected for analysis of the total protein content of the sample and another was collected for analysis of several cellular parameters. The supernatants were used to determine the rate of L-lactate production and the pellets were used for the other analytical procedures.

The cellular parameters determined were the percentages of viability, altered acrosomes and total motility. The first two parameters were determined by counting 200–300 spermatozoa at × 1000 magnification and staining by double Trypan blue–Giemsa stain, as described by Rodríguez-Gil *et al.* (1994). The percentage of total motility was defined as the percentage of spermatozoa that showed a curvilinear velocity (the instantaneously recorded sequential progression along the whole trajectory of the spermatozoon) > 20 µm s^{-1} (see Mogas *et al.*, 1998). A computerized analytical system was used to determine motility (sperm class analyzer; Microptic, Barcelona). A 5 µl sperm sample was placed on a prewarmed (37°C) siliconized microslide and covered with a 25 mm^2 siliconized coverslip. Observations were made at × 200 magnification on a negative phase-contrast microscope with a warmed stage (37°C).

For determination of glucose 6-phosphate, fructose 6-phosphate and ATP content, frozen samples were homogenized by sonication in 300 µl of 10% (v/v) ice-cold perchloric acid. Homogenates were centrifuged at 10 000 ***g*** for 15 min at 4°C, and the supernatants were neutralized with 5 mol K_2CO_3 l^{-1} before analysis.

For determination of ribose 5-phosphate content, frozen samples were homogenized by sonication in 300 µl of ice-cold 8% (v/v) trichloroacetic acid. Homogenates were centrifuged at 10 000 ***g*** for 15 min at 4°C. The samples were neutralized with 10 mol NaOH l^{-1}. A further 33 µl of 10 mol NaOH l^{-1} was added to the neutralized homogenates for 10 min at 25°C and, finally, 33 µl of 10 mol HCl l^{-1} was added to reach pH 6.5. This procedure removes traces of ketopentose phosphates that could contaminate samples. Analyses were performed on these samples.

Frozen samples were homogenized by sonication with 300 µl of ice-cold 30% (w/v) KOH and heated at 100°C for 15 min. Glycogen content was determined in these extracts.

Whole, fresh sperm-rich fractions of the ejaculate were centrifuged immediately at 600 ***g*** for 10 min to determine total hexokinase activity. The seminal plasma was discarded and the resultant pellets were frozen immediately in liquid nitrogen, and stored at –80°C until analysis. Total hexokinase acitvity was determined in thawed samples that had been

homogenized by sonication in 250 µl of an ice-cold buffer (pH 7.4) containing 500 mmol glycilglycine l^{-1}, 2 mol KCl l^{-1}, 100 mmol dithiothreitol l^{-1}, 300 iu aprotinin ml^{-1} and 100 mmol phenylmethylsulphonyl fluoride l^{-1} (homogenation buffer). Homogenized samples were centrifuged at 10 000 ***g*** for 15 min at 4°C. Hexokinase activity was measured in the resultant supernatants and in the pellets, which were washed once in 500 µl of the homogenation buffer to eliminate the remaining hexokinase activity. Finally, the pellets were resuspended in 250 µl homogenation buffer.

For determination of the rate of CO_2 production, pooled ejaculates were processed as above, and 500 µl aliquots of the final cellular suspension in the Krebs–Ringer medium were incubated for 30 min at 37°C in stoppered vials in the presence of increasing concentrations of either [U^{14}C]glucose or [U^{14}C]fructose. At the end of the incubations, a piece of filter paper soaked with 200 µl phenethylamine was placed into each of the vials, avoiding direct contact with the suspension. After addition of 85 µl of 10% (v/v) $HClO_4$ to the cell suspension, the vials were shaken for another 30 min, and the radioactivity in the filter papers was counted. Before addition of the radioactive material, a 10 µl aliquot of the cell suspension was taken to determine total protein content.

Analytical procedures

The glucose 6-phosphate concentration was determined enzymatically as described by Michal (1984), after adaptation of the method to a Cobas Bio autoanalyser. The batch of glucose 6-phosphate dehydrogenase used for the analysis (Boehringer Mannheim, Mannheim) was tested for the absence of contaminant hexose 6-phosphate isomerase activity, which could increase glucose 6-phosphate content. Only batches of glucose 6-dehydrogenase without significant hexose 6-phosphate isomerase activity were used. After determination of glucose 6-phosphate, the reaction mixture was added to 500 iu hexose 6-phosphate isomerase ml^{-1} (Boehringer Mannheim) and the mixture was incubated to determine the total hexose 6-phosphate content. Fructose 6-phosphate content was obtained after subtraction of the glucose 6-phosphate content from the total values, assuming that practically all of the hexose phosphates that were not glucose 6-phosphate were fructose 6-phosphate.

The concentrations of L-lactate (Noll, 1984), ribose 5-phosphate (Racker, 1984), glycogen (Ballester *et al.*, 2000) and ATP (Lambrecht and Trantschold, 1984), and total hexokinase activity (Otaegui *et al.*, 2000), were determined after adaptation to a Cobas Bio autoanalyser.

Total protein content of the samples was determined by the Bradford method (Bradford, 1976), using a commercial kit (Bio-Rad Laboratories, Hercules, CA).

Immunological techniques

Western blot analyses were performed on dog spermatozoa that had been homogenized by sonication in ice-cold 10 mmol Tris–HCl buffer l^{-1} (pH 7.4) containing 1% (w/v) dodecyl sodium sulphate (SDS) and 1 mmol Na_2VO_4 l^{-1} (proportion 1:5, v/v) to avoid changes in the overall phosphorylation status of the homogenates. The samples were briefly boiled using a microwave and were centrifuged at 10 000 ***g*** for 15 min at 4°C. Western blot analysis of spermatozoa was based on SDS gel electrophoresis (Laemmli, 1970), followed by transfer to nitrocellulose (Burnett, 1981). The transferred samples were tested with the antibodies at a dilution (v/v) of 1: 500 (anti Glut 3), 1:1000 (anti-Glut 5 and anti-phosphotyrosine PY20), 1: 2000 (anti-SGLT-1) and 1: 4000 (anti-Glut 1 and anti-Glut 2). Immunoreactive proteins were tested using peroxidase-conjugated anti-rabbit secondary antibody (Amersham) and the reaction was developed with an ECL-Plus detection system (Amersham).

Human spermatozoa used as positive controls in some western blot analyses were obtained from anonymous volunteers from the Autonomous University of Barcelona. Extracts from Chinese hamster ovary (CHO) cells and from the small intestine, kidney and muscle of rats were obtained from the laboratory of J. J. Guinovart (University of Barcelona). These extracts were also used as positive controls for some western blot analyses.

Immunocytochemistry was performed with sperm cells seeded onto gelatin-coated glass coverlips (10 mm × 10 mm). The spermatozoa 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 $NaBH_4$ ml^{-1} to eliminate autofluorescence, and blocked in 3% (w/v) bovine serum albumin in PBS. The spermatozoa were incubated with the antibodies (dilution 1: 500, 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). Fluorescence images were obtained by a Leica TCS 4D confocal scanning laser microscope (Leica Lasertechnik, Heidelberg), adapted to an inverted Leitz DMIRBE microscope and a × 63 (NA 1,4 oil) Leitz Plan-Apo Lens (Leitz; Stuttgart). The light source was an argon/krypton laser (75 mW).

Statistical analyses

When stated, putative significance of differences was calculated by either two-way ANOVA (in non-paired data) or by the Student's *t* test (in paired results).

Results

Variations in the percentages of total motility, viability and altered acrosomes in dog spermatozoa incubated in the presence or absence of fructose and glucose

Incubation of spermatozoa in a sugar-free medium induced a progressive and slight decline in their overall function, as shown by the slight decrease in the percentages of total motility and viability and the increase in the

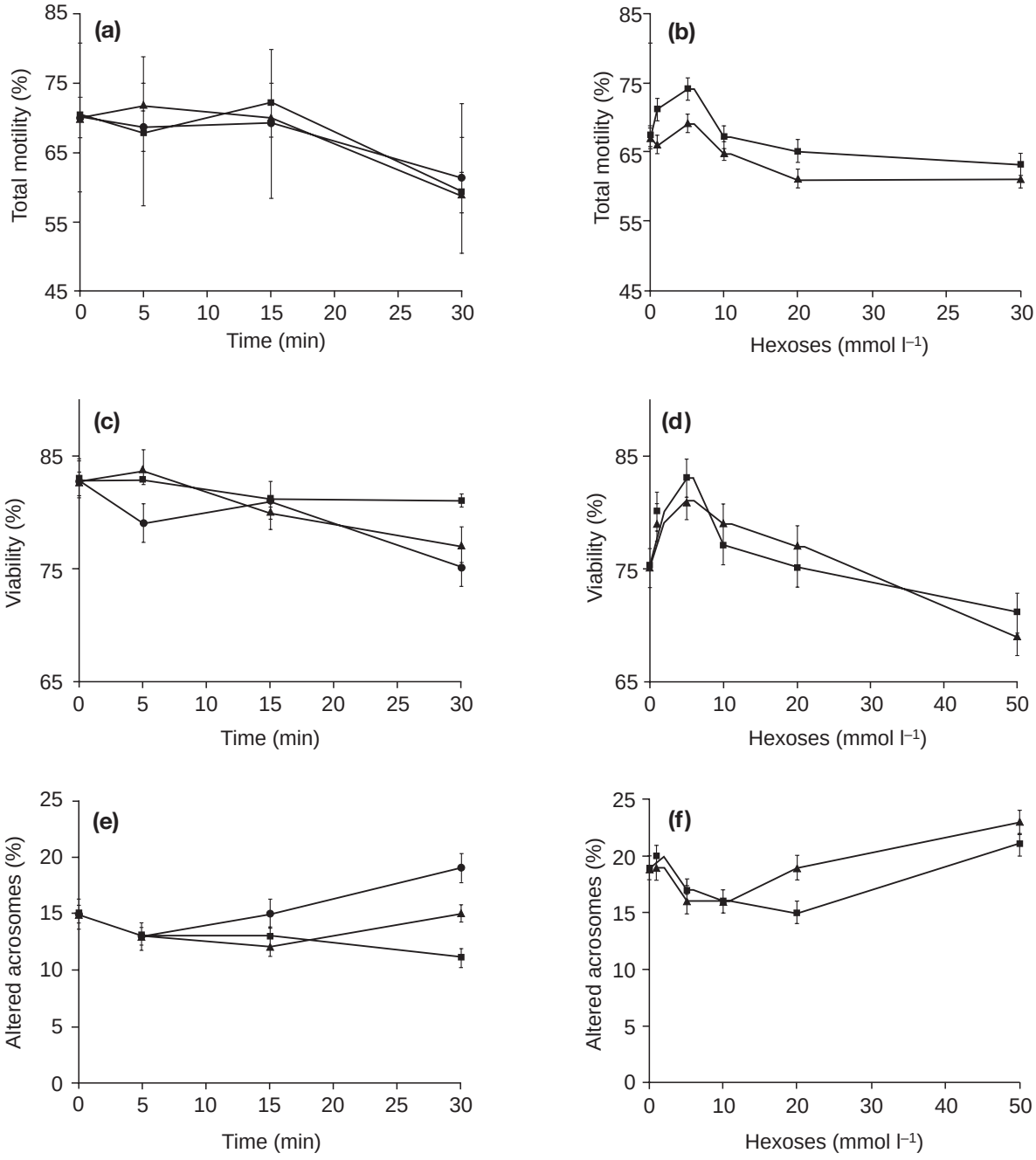

Fig. 1. Time- and concentration-dependent effects of glucose and fructose on the percentages of total motility, viability and altered acrosomes in fresh dog spermatozoa. (a,c,e) Spermatozoa were incubated in the absence (●) or presence of 10 mmol glucose l^{-1} (▲) or 10 mmol fructose l^{-1} (■) and, at the times indicated, aliquots were taken to determine the percentages of (a) total motility, (c) viability and (e) altered acrosomes. (b,d,f) Spermatozoa were incubated for 15 min with increasing concentrations of glucose (▲) or fructose (■) and aliquots were taken to determine the percentages of (b) total motility, (d) viability or (f) altered acrosomes. Data are means ± SEM for 11 separate experiments.

percentage of altered acrosomes after 30 min of incubation (Fig. 1). The addition of either 10 mmol glucose l^{-1} or 10 mmol fructose l^{-1} had little effect. Only after 30 min of incubation did glucose and, partially, fructose, recover percentage viability and altered acrosomes (but not total motility), reaching values similar to those observed at the beginning of the incubations (Fig. 1). These small effects were observed at sugar concentrations from 1 mmol l^{-1} to 10 mmol l^{-1}, whereas higher concentrations had a deleterious effect on spermatozoa, with a clear decrease in the percentages of

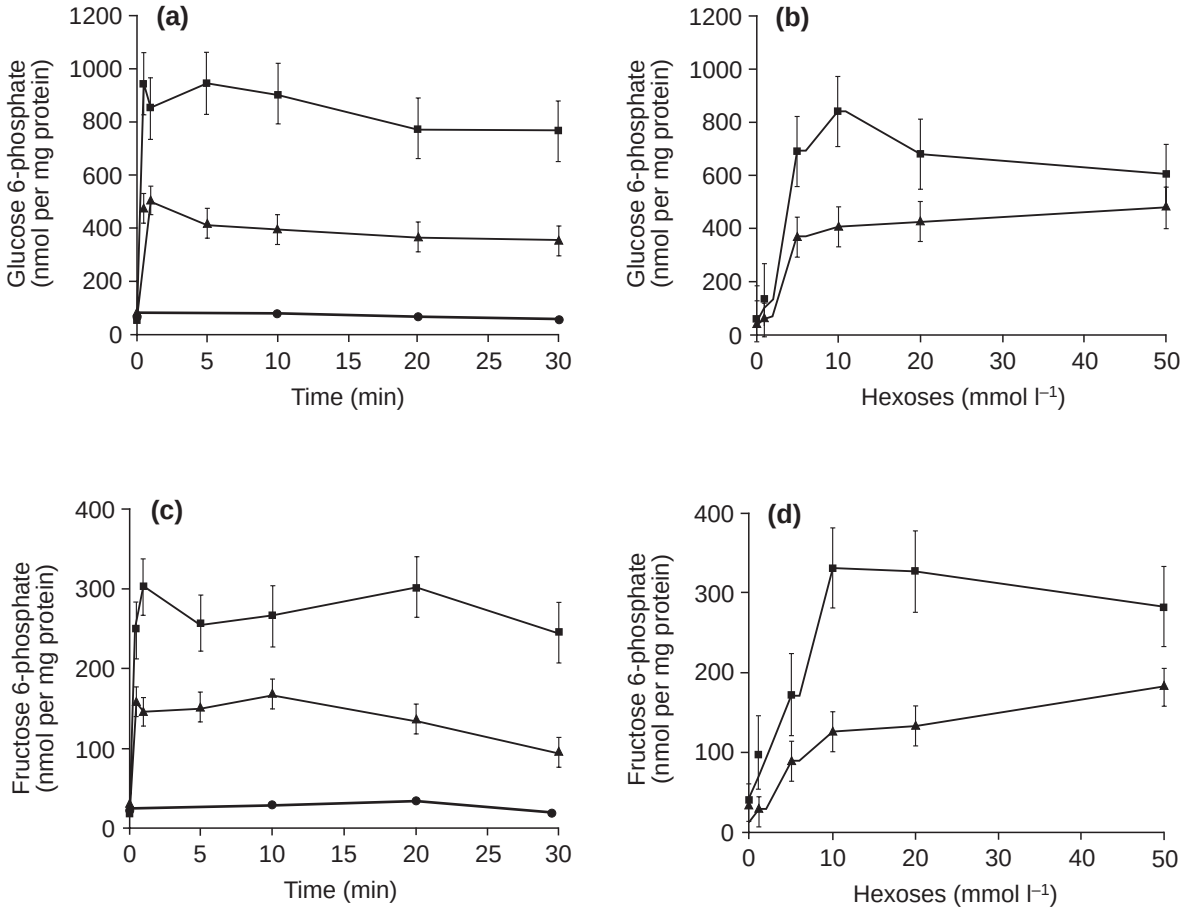

Fig. 2. Time- and concentration-dependent effects of glucose and fructose on intracellular content of glucose 6-phosphate and fructose 6-phosphate in fresh dog spermatozoa. (a,c) Spermatozoa were incubated in the absence (●) or presence of 10 mmol glucose l^{-1} (▲) or 10 mmol fructose l^{-1} (■) and, at the times indicated, aliquots were taken to determine the intracellular concentrations of (a) glucose 6-phosphate and (c) fructose 6-phosphate. (b,d) Spermatozoa were incubated for 10 min with increasing concentrations of glucose (▲) or fructose (■) and aliquots were taken to determine the intracellular contents of (b) glucose 6-phosphate or (d) fructose 6-phosphate. Data are means ± SEM for 11 separate experiments.

total motility and viability, and an increase in the percentage of altered acrosomes (Fig. 1). Thus, incubation in the absence of sugars for at least 30 min had a slight deleterious effect on dog spermatozoa and the addition of either glucose or fructose counteracted this impairment at least partially.

Effects of glucose and fructose on intracellular content of glucose 6-phosphate and fructose 6-phosphate in dog spermatozoa from fresh ejaculates

Incubation of fresh semen with 10 mmol glucose l^{-1} induced a very large, rapid and significant ($P < 0.05$) increase in intracellular glucose 6-phosphate content, which increased from about 80 nmol per mg protein immediately before incubation to about 500 nmol per mg protein at only 30 s after addition of the sugar (Fig. 2a). This increase was maintained for about 30 min and glucose 6-phosphate content after 30 min was about 400 nmol per mg protein (Fig. 2a). The addition of 10 mmol fructose l^{-1} induced an even greater increase in glucose 6-phosphate content, which increased from about 80 nmol per mg protein to about 950 nmol per mg protein after only 30 s of incubation. These values were maintained throughout incubation (about 800 nmol per mg protein after 30 min of incubation; Fig. 2a).

The effects of these monosaccharides on glucose 6-phosphate content were dose-dependent, although they differed in some aspects. The maximal effect of glucose (glucose 6-phosphate content of about 400 nmol per mg protein) was observed at concentrations as low as 5 mmol l^{-1} (Fig. 2b). Concentrations > 5 mmol l^{-1} did not increase the effect of the sugar and doses of 1 mmol l^{-1} hardly modified glucose 6-phosphate content compared with spermatozoa incubated without sugars (Fig. 2b). In contrast, the effect of fructose increased at concentrations

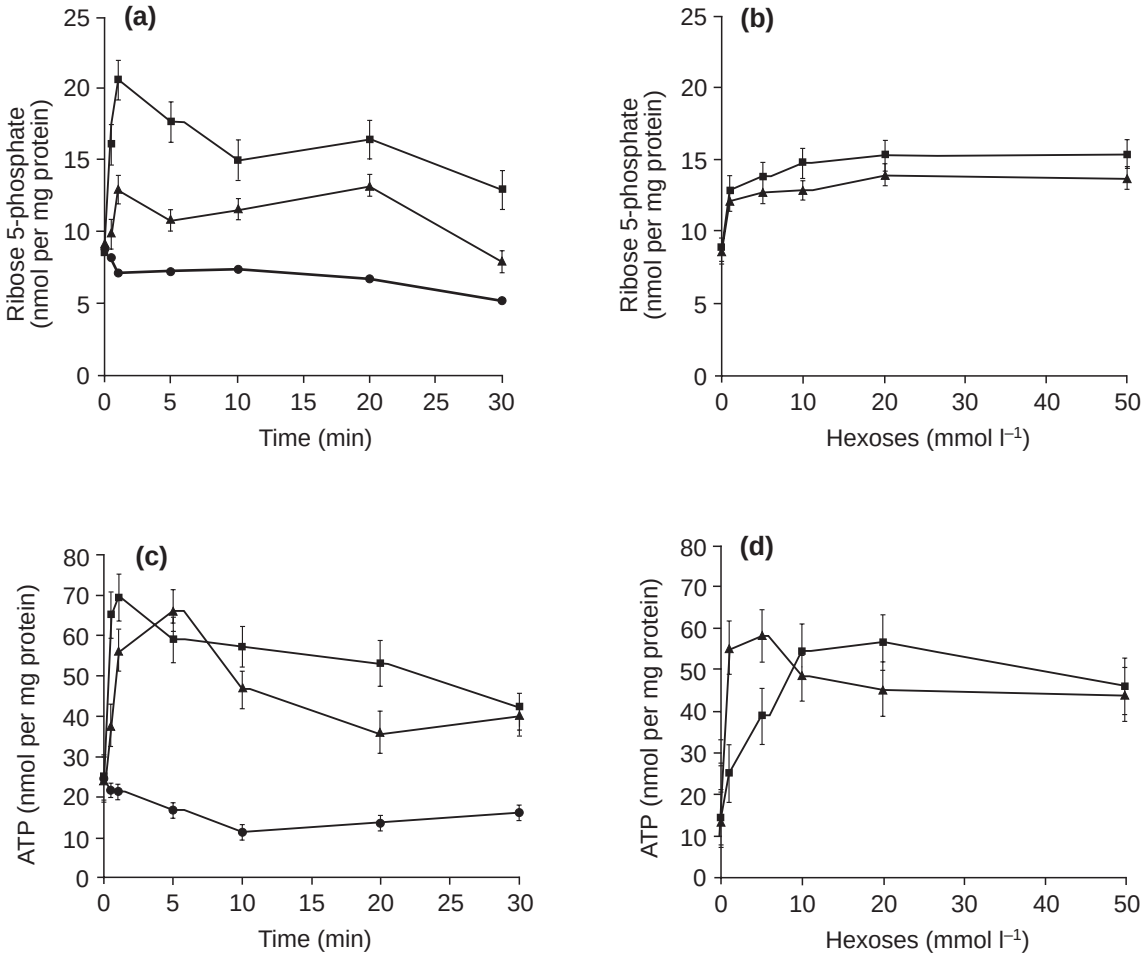

Fig. 3. Time- and concentration-dependent effects of glucose and fructose on intracellular content of ribose 5-phosphate and ATP in fresh dog spermatozoa. (a,c) Spermatozoa were incubated in the absence (●) presence of 10 mmol glucose l^{-1} (▲) or 10 mmol fructose l^{-1} (■) and, at the times indicated, aliquots were taken to determine the intracellular concentrations of (a) ribose 5-phosphate or (c) ATP. (b,d) Spermatozoa were incubated for 10 min with increasing concentrations of glucose (▲) or fructose (■) and aliquots were taken to determine the intracellular contents of (b) ribose 5-phosphate or (d) ATP. Data are means ± SEM for 11 separate experiments.

> 1 mmol l^{-1}, and peaked (glucose 6-phosphate content of about 900 nmol per mg protein) at 10 mmol l^{-1} (Fig. 2b).

The effects of glucose and fructose on fructose 6-phosphate content were also intense and rapid, and, again, fructose had a much greater and significant ($P < 0.05$) effect. Fructose 6-phosphate content increased from about 25 nmol per mg protein in control sperm cells to about 150 nmol per mg protein after only 30 s of incubation with 10 mmol glucose l^{-1}. These values were maintained after 30 min of incubation (Fig. 2c). Addition of 10 mmol fructose l^{-1} also increased fructose 6-phosphate values to about 300 nmol per mg protein after 30 s of incubation, which were maintained for 30 min (Fig. 2c).

Similar to the intracellular glucose 6-phosphate content, the effect of glucose on fructose 6-phosphate content was observed at concentrations > 1 mmol l^{-1} and reached maximal effect (about 120 nmol per mg protein versus about 30 nmol per mg protein in spermatozoa incubated without sugars) at doses ⩾ 10 mmol (Fig. 2d). Addition of fructose induced an increasing effect at doses as low as 1 mmol l^{-1}, although a maximal effect (about 330 nmol per mg protein) was obtained from 10 mmol l^{-1} upwards (Fig. 2d).

Effects of glucose and fructose on intracellular ribose 5-phosphate content in dog spermatozoa from fresh ejaculates

Dog spermatozoa from fresh ejaculates showed a ribose 5-phosphate content of about 7 nmol per mg protein. This value decreased slightly after incubation in a sugar-free medium, reaching about 5 nmol per mg protein after 30 min (Fig. 3a). Incubation in a medium containing 10 mmol glucose l^{-1} induced a fast and significant ($P < 0.05$) increase

Table 1. Time and concentration effects of glucose and fructose on intracellular glycogen content of dog spermatozoa

	Time of incubation (min)			
Treatment	0	5	15	30
Without sugars	0.24 ± 0.02	0.22 ± 0.02	0.20 ± 0.02	0.17 ± 0.01
10 mmol glucose l^{-1}	0.24 ± 0.02	0.25 ± 0.02	0.27 ± 0.02	0.32 ± 0.02
10 mmol fructose l^{-1}	0.24 ± 0.02	0.29 ± 0.02	0.39 ± 0.02	0.47 ± 0.02

	Hexose (mmol l^{-1})					
Treatment	0	1	5	10	20	50
30 min glucose	0.16 ± 0.01	0.18 ± 0.01	0.22 ± 0.02	0.29 ± 0.02	0.35 ± 0.03	0.41 ± 0.03
30 min fructose	0.18 ± 0.02	0.25 ± 0.02	0.34 ± 0.02	0.41 ± 0.02	0.54 ± 0.03	0.63 ± 0.03

Data are means ± SEM for 11 separate experiments. Glycogen content is expressed as μmol glucose per mg protein.

in this parameter, reaching about 12 nmol per mg protein after 1 min (Fig. 3a), which was maintained for 20 min, and then decreased to about 8 nmol per mg protein after 30 min (Fig. 3a). Addition of 10 mmol fructose l^{-1} induced a much greater and also significant ($P < 0.05$) increase in ribose 5-phosphate content, which reached > 20 nmol per mg protein after 1 min and a progressive decrease, reaching about 13 nmol per mg protein, after 60 min (Fig. 3a).

Increasing the concentrations of the monosaccharides in the medium did not change their effect on spermatozoa. Thus, incubation with 1 mmol glucose l^{-1} for 10 min induced an increase in ribose 5-phosphate content to about 12 nmol per mg protein (Fig. 3b). These values did not change significantly for concentrations of glucose up to 50 mmol l^{-1} (Fig. 3b). A similar effect was observed with fructose, although in this case the ribose 5-phosphate content was slightly higher (Fig. 3b).

Effects of glucose and fructose on intracellular glycogen content in dog spermatozoa

Incubation of dog spermatozoa in a sugar-free medium induced a progressive decrease in the intracellular glycogen content, which ranged from 0.24 ± 0.02 μmol glucose per mg protein in the initial samples (which were taken about 10 min after the washing without sugars) to 0.17 ± 0.01 μmol glucose per mg protein after 30 min of incubation (Table 1). Incubation with 10 mmol glucose l^{-1} did not modify this parameter significantly after 15 min of incubation, and only after 30 min of incubation did it increase significantly ($P < 0.05$) (0.32 ± 0.02 μmol glucose per mg protein; Table 1). Incubation with 10 mmol fructose l^{-1} induced a rapid and significant ($P < 0.05$) increase in glycogen content, which increased to 0.39 ± 0.02 μmol glucose per mg protein after only 15 min of incubation, and reached values of 0.47 ± 0.02 μmol glucose per mg protein after 30 min (Table 1). There was also a concentration-dependent effect of the two sugars on glycogen content, and both reached their maximal effects at concentrations of 50 mmol l^{-1} (Table 1). Notwithstanding, fructose had a much greater and more significant ($P < 0.05$) effect on glycogen (0.63 ± 0.03 versus 0.41 ± 0.03 μmol glucose per mg protein after 30 min of incubation with 50 mmol glucose l^{-1}; Table 1).

Effects of glucose and fructose on L-lactate production in dog spermatozoa

Dog spermatozoa incubated in the absence of sugars did not produce significant amounts of extracellular L-lactate (Table 2). Incubation with 10 mmol glucose l^{-1} induced measurable extracellular concentrations of L-lactate, which increased progressively, reaching 1.97 ± 0.01 μmol per mg protein after 60 min (Table 2). Incubation with 10 mmol fructose l^{-1} induced a significant ($P < 0.05$), higher rate of L-lactate production, reaching 3.53 ± 0.02 μmol per mg protein after 30 min of incubation (Table 2). The greater effect of fructose was also observed when spermatozoa were incubated for 30 min with a concentration of monosaccharides ranging from 1 mmol l^{-1} to 50 mmol l^{-1} (Table 2).

Effects of glucose and fructose on intracellular ATP content in dog spermatozoa

Dog spermatozoa incubated in the absence of sugars showed a progressive decrease in intracellular content of ATP, which decreased from about 25 nmol per mg protein at the start of incubation to about 15 nmol per mg protein after 60 min (Fig. 3c). The addition of 10 mmol glucose l^{-1} induced a large and rapid increase in ATP, reaching about 65 nmol per mg protein after 5 min, which was followed by a decrease in ATP content, reaching about 40 nmol per mg protein after 30 min (Fig. 3c). Incubation with 10 mmol fructose l^{-1} induced a faster and greater increase in ATP content, reaching about 70 nmol per mg protein after 1 min of incubation (Fig. 3c). This increase was also followed

Table 2. Effects of incubation with glucose or fructose on the rate of L-lactate formation in dog spermatozoa

	Time of incubation (min)			
Treatment	0	5	15	30
Without sugars	0.01 ± 0.01	0.01 ± 0.01	0.11 ± 0.01	0.27 ± 0.01
10 mmol glucose l^{-1}	0.01 ± 0.01	0.24 ± 0.01	1.01 ± 0.01	1.97 ± 0.01
10 mmol fructose l^{-1}	0.01 ± 0.01	0.49 ± 0.01	2.01 ± 0.02	3.53 ± 0.02

	Hexose (mmol l^{-1})					
Treatment	0	1	5	10	20	50
30 min glucose	0.25 ± 0.01	0.82 ± 0.01	1.01 ± 0.01	1.99 ± 0.01	2.24 ± 0.02	2.76 ± 0.02
30 min fructose	0.27 ± 0.02	1.14 ± 0.01	2.01 ± 0.02	3.40 ± 0.02	4.23 ± 0.03	5.15 ± 0.03

Data are means ± SEM for 11 separate experiments. The amount of L-lactate is expressed as μmol per mg protein.

Table 3. Effects of glucose and fructose on the rate of CO_2 production in dog spermatozoa

	Hexose (mmol l^{-1})				
Treatment	1	5	10	20	50
30 min glucose	18.7 ± 0.9	42.8 ± 1.9	56.2 ± 2.5	90.0 ± 3.9	144.3 ± 5.9
30 min fructose	48.7 ± 2.2	97.5 ± 4.5	105.0 ± 4.9	120.1 ± 5.2	180.7 ± 7.1

Data are means ± SEM for 11 separate experiments. The CO_2 production is expressed as nmol per mg protein.

by a progressive decrease in ATP content, reaching about 50 nmol per mg protein after 30 min of incubation (Fig. 3c).

The effects of glucose and fructose on ATP content were concentration-dependent. The maximal effect of the former was reached at about 1–5 mmol l^{-1} of the sugar (about 55 nmol per mg protein by incubating spermatozoa with 5 mmol glucose l^{-1} for 10 min). Higher concentrations of glucose induced a less intense increase in ATP content. In contrast, the maximal effect of fructose was observed at concentrations of 10–50 mmol l^{-1}, with a much weaker effect than glucose at concentrations of 1–5 mmol l^{-1} (Fig. 3d).

Effects of glucose and fructose on CO_2 production in dog spermatozoa

There was a clear concentration-dependent effect of both glucose and fructose on CO_2 production. Thus, incubation with increasing concentrations of glucose for 30 min induced a progressive increase in CO_2 production, with maximal production (about 180 nmol per mg protein) at 50 mmol l^{-1} (Table 3). In contrast, incubation for 30 min with lower concentrations of fructose produced a much more intense and significant ($P < 0.05$) effect on CO_2 production, reaching values of 97.5 ± 0.06 nmol per mg protein with 5 mmol fructose l^{-1} compared with 42.8 ± 0.04 nmol per mg protein with 5 mmol glucose l^{-1} (Table 3). This greater effect of fructose was maintained even at the higher concentrations used (180.7 ± 7.1 nmol per mg protein with 50 mmol fructose l^{-1} versus 144.3 ± 5.9 nmol per mg protein with 50 mmol glucose l^{-1}; Table 3).

Modulation of total hexokinase activity from crude dog sperm extracts by glucose and fructose

There was a concentration-dependent effect of glucose on total hexokinase activity in both supernatants and pellet extracts from fresh dog ejaculates. Maximal activity was reached at glucose concentrations of about 3 mmol l^{-1} in both fractions (about 7 mU per mg protein in supernatants and about 5 mU per mg protein in resuspended pellets; Fig. 4a,b). In contrast, fructose had a much more intense effect on the hexokinase activity of supernatants. In this case, the maximal effect (about 10 mU per mg protein) was reached at concentrations as low as 0.5 mmol l^{-1}, indicating a greater sensitivity for fructose (Fig. 4a). Differences in hexokinase activity between fructose and glucose were significant ($P < 0.05$). Furthermore, there was low fructose-linked hexokinase activity in pellets, only reaching detectable values at concentrations of about 3 mmol l^{-1} (Fig. 4b).

Effects of glucose and fructose on the tyrosine phosphorylation pattern of dog spermatozoa

Dog sperm extracts (total protein content by electrophoretic lane: 30 μg) from fresh ejaculates showed a faint, but well-defined, specific tyrosine phosphorylation pattern

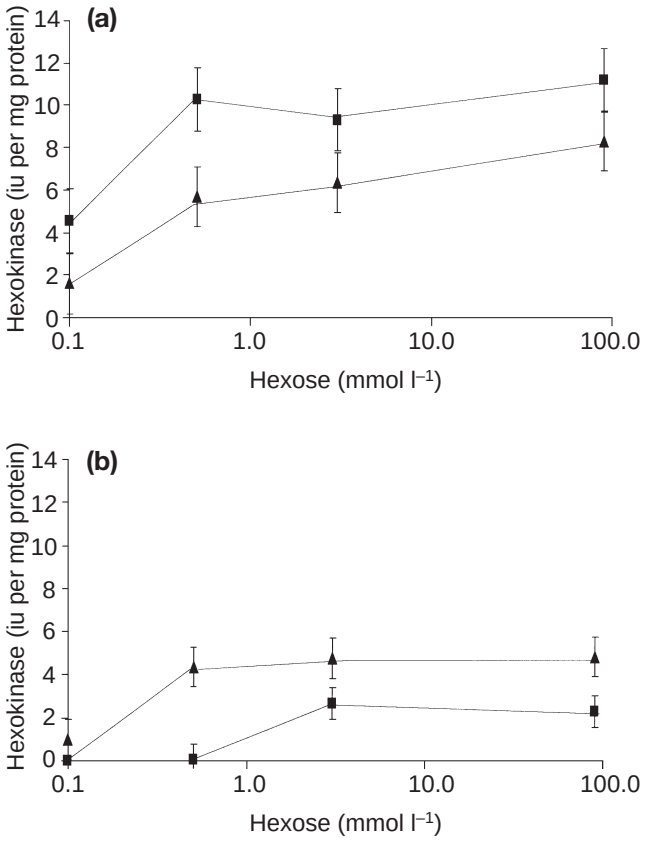

Fig. 4. Concentration-dependent effects of glucose and fructose on the total hexokinase activity of crude sperm extracts. The total hexokinase activity of (a) supernatants and (b) resuspended pellets obtained from crude extracts of fresh ejaculated dog spermatozoa was evaluated in the presence of increasing concentrations of glucose (▲) or fructose (■). Data are means ± SEM for 11 separate experiments.

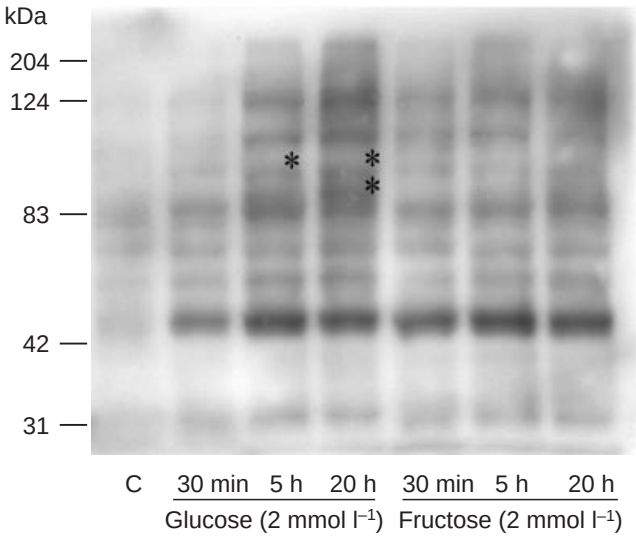

Fig. 5. Tyrosine phosphorylation pattern of pooled dog spermatozoa from four ejaculates incubated with 2 mmol glucose l^{-1} or 2 mmol fructose l^{-1}. Aliquots were collected at the times indicated to determine the tyrosine phosphorylation pattern after a western blot analysis against an anti-mouse phosphorylated tyrosine antibody. The amount of total protein analysed in each lane was 30 µg. A representative western blot showing eight separate experiments is shown. C: samples incubated without sugars for 5 min. Glucose (2 mmol l^{-1}): incubation with 2 mmol glucose l^{-1}. Fructose (2 mmol l^{-1}): incubation with 2 mmol fructose l^{-1}. Asterisks indicate some glucose-induced, phosphorylated proteins that were only slightly phosphorylated after fructose incubation.

(Fig. 5). Incubation with 2 mmol glucose l^{-1} induced a rapid increase in the intensity of this pattern. The increase in phosphorylation was apparent after only 30 s of incubation and maximal intensity was reached after 5 min. This effect was maintained for at least 20 min of incubation (Fig. 5). Incubation with 10 mmol glucose l^{-1} induced a similar effect (data not shown). An increase in the intensity of tyrosine phosphorylation was also observed with 2 mmol fructose l^{-1}. However, the fructose-induced phosphorylation pattern differed slightly from that of glucose. Thus, incubation with fructose did not induce a marked increase in the phosphorylation of a specific protein with a molecular mass of about 90 kDa (Fig. 5). Moreover, several proteins with molecular masses ranging from 85 kDa to 125 kDa were more phosphorylated with glucose than with fructose (Fig. 5). The effect of fructose was also rapid, as it was detectable after only 30 s of incubation and lasted for at least 20 min (Fig. 5). Incubation with 10 mmol fructose l^{-1} gave similar results to incubation with 2 mmol fructose l^{-1} (data not shown).

Expression of hexose transporters in dog spermatozoa

Dog sperm extracts (total protein content by electrophoretic lane: 30 µg) showed several hexose transporters. Western blot analysis revealed a protein that reacted against the anti-Glut 3 antibody in spermatozoa from men and dogs (Fig. 6a). This protein has a molecular mass of about 45 kDa, which matches that observed in CHO cell cultures, which were used as positive controls, and is also consistent with the report of Asano *et al.* (1992). Our results indicate that the hexose transporter Glut 3 is present in dog spermatozoa.

In addition, the western blot analysis performed against the Glut 5 antibody showed a positive signal in dog and human spermatozoa, although in this case there were some differences. Human samples (Burant *et al.*, 1992) had a positive signal of about 40 kDa, whereas dog sperm cells had two positive bands, at about 35 kDa and 45 kDa. However, as Glut transporters show distinct molecular masses depending on their phosphorylation status, as well as the absence of a positive signal in the control lane of rat muscle (Fig. 6b), it can be assumed that these signals are compatible with the presence of Glut 5 in dog spermatozoa.

The analysis performed on the SGLT-1 transporter also showed two positive signals of about 50–60 kDa, which

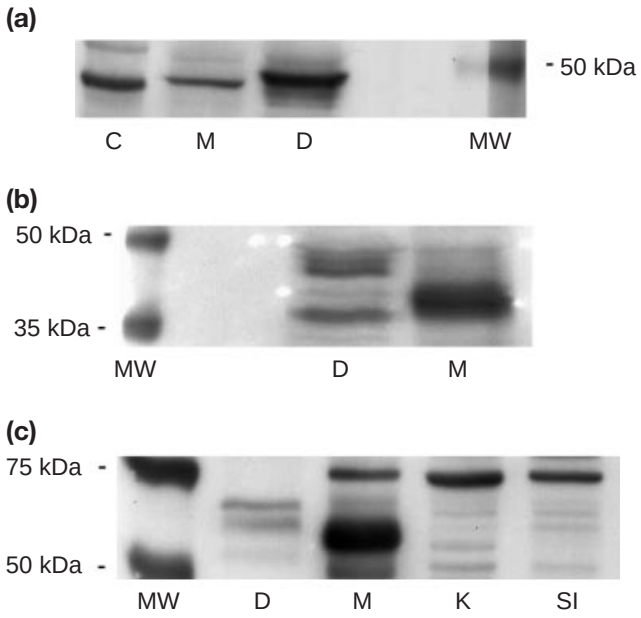

Fig. 6. Western blot analyses of hexose transporters in fresh dog spermatozoa. (a) A representative western blot using an anti-rat Glut 3 polyclonal antibody, showing the positive reaction of dog sperm (D), the two positive controls based on extracts from Chinese hamster ovary culture cells (C) and on human sperm (M). (b) A representative western blot using an anti-rat Glut 5 polyclonal antibody, showing the positive reaction of dog sperm (D), with extracts from human sperm (M) used as a positive control. (c) A representative western blot using an anti-rat SGLT-1 polyclonal antibody, showing the positive reaction of dog sperm (D); rat extracts from the small intestine (SI), kidney (K) and muscle (M) were used as positive controls. MW: molecular weight markers.

may correspond to two proteins or to one protein with a distinct degree of phosphorylation glycosylation (Fig. 6c). The 50 kDa signal was similar to that observed in extracts from rat muscle, which roughly corresponds to the low-affinity, phloretin-sensitive Na^+/glucose cotransporter SAAT-1/SGLT2 (Mackenzie *et al.*, 1994). Moreover, the muscle, small intestine and kidney of rat showed another band of about 75 kDa, which corresponds to the SGLT-1 transporter (Fig. 6c). As there is a close structural relationship between the proteins of the SGLT family (Mackenzie *et al.*, 1996), recognition of all of these proteins by the same polyclonal antibody is likely, thereby indicating that the sperm proteins which recognize the SGLT-1 antibody are probably members of the SGLT family of transporters.

No positive or conclusive signals were observed when sperm extracts were tested against anti-Glut 1, anti-Glut 2 and anti-Glut 4 (data not shown).

Immunolocation of Glut 3 showed that this transporter was located mainly at the mid-piece (Fig. 7a). In contrast, Glut 5 was located mainly at the peri-acrosomal region and mid-piece (Fig. 7b), whereas the antibody against SGLT-1 showed a positive signal in the peri-acrosomal region, the equatorial zone and the mid-piece (Fig. 7c).

Discussion

The results of the present study indicate that the distinct functional effects of glucose and fructose are the result of differences in the mechanisms that maintain the equilibrium in dog sperm energy status. In this regard, the net ATP content of dog spermatozoa, which is strongly related to energy status, is the result of a dynamic equilibrium between its formation and its degradation. Our results show that fructose increases the rate of the metabolic pathways and, hence, ATP formation, more than glucose. This metabolic 'push', which is initiated by a very large and rapid increase in glucose 6-phosphate content, can be inferred by observing the effect of fructose on the rate of production of L-lactate and CO_2, and on glycogen deposition. Moreover, the increase in ribose 5-phosphate content, which was even greater than that of glucose, is secondary to the increase in glucose 6-phosphate. However, fructose may increase the rate of ATP consumption in at least two ways. Firstly, by increasing the motility-related consumption of ATP. Thus, incubation with fructose results in a specific motility pattern, which is more rapid and linear than that observed after incubation with glucose (Rigau *et al.*, 2001). As most sperm energy consumption is devoted to maintenance of motility (Roldán, 1998), the effect of fructose on motility is probably related to an increase in the rate of ATP consumption. The second mechanism of ATP consumption is related to sperm phosphorylating activity. Fructose induces a very strong increase in the rate of hexose phosphorylation with respect to glucose, as shown by the results of glucose 6-phosphate and fructose 6-phosphate. This increase, summing up the increase in the overall protein tyrosine phosphorylation, could lead to the establishment of substrate cycling, as described by Hammersted and Lardy (1983), which consumes ATP. This substrate cycling is a physiological characteristic of mammalian spermatozoa, as they rarely achieve the theoretical stoichiometric ATP yield of the glycolytic pathway (Hammersted and Lardy, 1983). Thus, the theoretical increase in ATP formation induced by incubation with fructose is counteracted by a concomitant increase in the motility-related ATP consumption and the restoration of relevant substrate cycling. The sum of these effects may explain the lack of clear differences in the net dog sperm ATP content after incubation with either glucose or fructose, although the effects of these sugars on energy status, and also on motility, differed greatly.

Despite their net, low ATP content, dog spermatozoa maintained a significant degree of motility regardless of the medium (Rigau *et al.*, 2001). This observation indicates that these cells have a very efficient system to maintain their function. Elaborate mechanisms of modulation of metabolism, such as a functional glycogen metabolism (Ballester *et al.*, 2000), act by optimizing the capacity of dog spermatozoa to perform their functions in an unfavourable environment by mechanisms such as maintenance of a constant intracellular ATP content over a long period of time. Therefore, long-term energy storage systems such as glycogen deposition

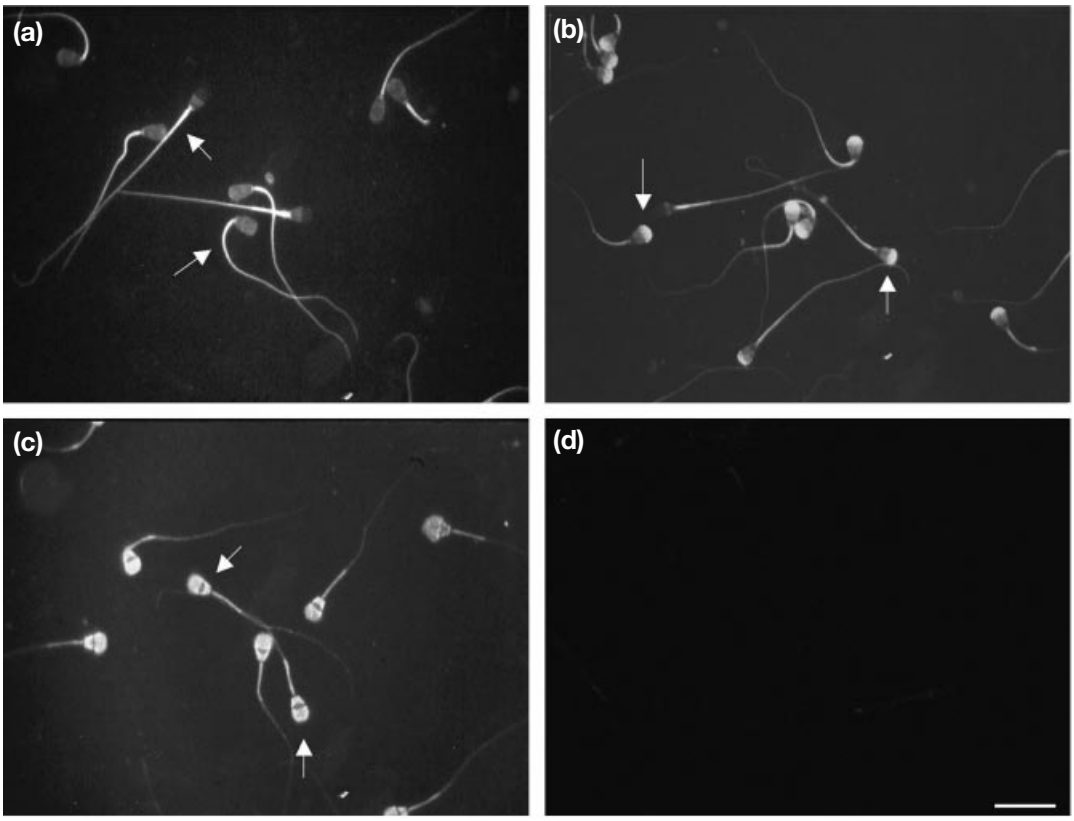

Fig. 7. Three-dimensional laser confocal images of the immunolocation of hexose transporters in fresh dog spermatozoa. Dog spermatozoa from fresh ejaculates were processed for immunocytochemical analysis in the presence of the (a) anti-Glut 3, (b) anti-Glut 5 or (c) anti-SGLT-1 antibodies, or (d) in the absence of antibodies. Arrows indicate the location of the positive signals. Scale bar represents 10 μm.

may form the basis of the maintenance of motility in an environment that induces very low intracellular ATP content.

Our results show that both glucose and fructose induce a rapid and strong increase in the overall protein tyrosine phosporylation of dog spermatozoa. An intriguing question remains as regards the physiological role that the sugar-induced increase in tyrosine phosphorylation has on dog spermatozoa. In mice, glucose-induced tyrosine phosphorylation is a crucial step in sperm capacitation and sperm–oocyte interaction (Visconti *et al.*, 1995a,b; Urner *et al.*, 2001). Similar results were not observed in dog spermatozoa in the present study. Nevertheless, an activating role for glucose and fructose on dog sperm function was observed, which is initiated by the rapid and intense increase in the tyrosine phosphorylation status of some specific proteins. This, in turn, could modulate critical sperm functions that must be modified immediately after ejaculation, namely sperm motility. Thus, the rapid changes in the motility pattern of dog spermatozoa that are induced by glucose and fructose (Rigau *et al.*, 2001) could be related to the changes in protein phosphorylation. Moreover, the slightly different patterns of tyrosine phosphorylation observed with fructose and glucose could be reflected in differential functional effects, such as the differential motility patterns observed after incubation with glucose or fructose (Rigau *et al.*, 2001). However, it is not surprising that incubation with sugars affects protein phosphorylation. Sugars modulate the activity of several enzymes in this way. For example, glucose and the phosphorylated metabolite glucose 6-phosphate modulate hepatic glycogen metabolism by either direct or indirect modification of the degree of phosphorylation of the key regulatory enzymes glycogen synthase and glycogen phosphorylase (Bollen *et al.*, 1998). Thus, several effects similar to those described for glycogen metabolism could form, at least partially, the basis of the action of glucose and fructose on the tyrosine phosphorylation pattern of dog spermatozoa, which almost certainly affects the function of these cells.

Our immunological analyses indicate that there are at least two hexose transporters in dog spermatozoa, namely Glut 3 and Glut 5. Moreover, the results against the SGLT-1 antibody indicate that dog spermatozoa have at least one protein that, although it is not SGLT-1, is included in the SGLT family of Na^+/voltage-dependent, phloretin-sensitive hexose and amino acid transporters. This observation can

be inferred by the presence of a protein that is reactive to the SGLT-1 antibody with a molecular mass similar to that of the SAAT-1/SGLT-2 antibody-sensitive protein from rat muscle (Mackenzie *et al.*, 1996). The crossreactivity of the proteins pertaining to the SGLT family to the polyclonal SGLT-1 antibody is due to the high sequence homology among these proteins, reaching values of about 90% (Mackenzie *et al.*, 1996).

Regarding the presence of Glut 3 and Glut 5 transporters in dog spermatozoa, our results are consistent with those reported for species such as bulls, mice and humans (Burant *et al.*, 1992; Angulo *et al.*, 1998). However, Glut 1, Glut 2 and Glut 4 transporters were not detected in dog spermatozoa and we can only speculate about the possible role of the SGLT-family proteins as hexose transporters. Therefore, we hypothesize that the main hexose transporters of dog spermatozoa are Glut 3 and Glut 5. The variety of transporters present in dog spermatozoa shows that these cells have a complex system for optimization of hexose transport and phosphorylation. In this regard, whereas Glut 3 is a very high affinity glucose transporter, Glut 5 shows a very high affinity for fructose (Mueckler, 1990). Thus, the presence of these two hexose transporters may indicate separate uptake pathways for glucose and fructose. These separate pathways may mark the starting point that leads to the differentiated functional effects of these sugars. Moreover, the observation of a differentiated hexose sensitivity of hexokinase activity in the supernatants and pellets of the dog sperm extracts indicates a follow-up in the differentiated pathways for fructose and glucose metabolism, which would be initiated at the sugar uptake step. Thus, it is hypothesized that the differential effects of these two monosaccharides on dog sperm function are initiated by a differentiated initial pathway of sugar uptake and, perhaps, phosphorylation. Moreover, the differentiated metabolic pathways for fructose and glucose lead to questions about the physiological role of fructose in dog spermatozoa. In many mammals, spermatozoa use glucose in preference to fructose when both substrates are available (Rikmenspoel and Caputo, 1966; Bedford and Hoskins, 1990; Jones and Connor, 2000). This may indicate that fructose has no relevant role in controlling the energy status of mammalian spermatozoa, as it is not the most efficient energy substrate for these cells. However, the presence of specific machinery for metabolizing fructose shows that this sugar is indeed playing such a role. At present we can only make speculations about this, although our results indicate that this substrate plays an activating and modulating role on dog sperm function more than a role as a mere passive energy substrate. Thus, the very fast and strong metabolic reaction that dog spermatozoa undergo after fructose incubation is compatible with a functional activation of the cells, which is mainly shown in the specific motility pattern obtained (Rigau *et al.*, 2001). This pattern differs greatly from that obtained after glucose incubation, which is less rapid and less linear (Rigau *et al.*, 2001). Other results, such as the presence of Glut 5, but not Glut 3, in the sperm head reinforce a specific, separate role for fructose. Similar results have been reported for other species (Angulo *et al.*, 1998). A separate location for glucose and fructose uptake could indicate a certain directionality of hexose metabolism. Thus, the presence of Glut 5 transporters in the peri-acrosomal region may indicate that the uptake of fructose by the head is needed but the reason remains unknown. In this respect, it is interesting to note that some ATP consumption occurs in the sperm head. Thus, the acrosome reaction is related to a cascade of phosphorylation– dephosphorylation reaction, which results in a net consumption of ATP (Roldán, 1998). In this sense, we cannot eliminate the possibility of the presence of a minor, semi-autonomous fructose-related, energy metabolism linked to the sperm head that may produce energy for local requirements.

In conclusion, these results indicate that the differences observed in the glucose and fructose metabolism of fresh dog spermatozoa could be mainly attributable to the differences in the phosphorylation rates of these sugars at concentrations of about 1–20 mmol l^{-1}. Due consideration must be given to this point when evaluating the changes in dog sperm function during their lifetime. Moreover, a full understanding of these differentiated functional effects is necessary when designing specific extenders to conserve dog spermatozoa in refrigeration for a long period of time.

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