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Lactate dehydrogenase activity of rat epididymis and spermatozoa: Effect of constant light

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SUMMARY

During its passage through the epididymis, the gamete undergoes a process of "maturation" leading to the acquisition of its fertilizing ability. The epididymis displays regional variations in the morphology and metabolic properties of its epithelium which are relevant for the progressive development of mature sperm characteristics. The epididymis has spontaneous peristaltic contractions and receives sympathetic innervation that is modulated by melatonin, a hormone synthesized and released by the pineal gland. Constant lighting disrupts melatonin synthesis and secretion. We have studied the effect of constant light on lactate dehydrogenase (LDH; EC 1.1.1.27) and its isozyme C4 activities and protein content in whole epididymis, epididymal tissue and in spermatozoa from caput and cauda segments. Animals were exposed from birth to an illumination schedule of 14 h light: 10 h dark (group L:D). At 60 days of age one group of animals was submitted to constant light over 50 days (group L:L). In order to test the fertilizing ability, the rats of each group were mated with soliciting estrous females. The percentage of pregnancies in females mated with males maintained in

L:L was remarkably lower than those in females mated with males maintained in the L:D photoperiod (44% and 88% respectively). Constant light increased protein concentration and LDH activity in caput as well as in cauda of total epididymis. On the contrary, in epididymal tissue, the protein content decreased in both epididymal sections compared with controls. When enzymatic activity was expressed in Units per spermatozoa, constant light induced a significant reduction of total LDH and LDHC₄ in caput and cauda spermatozoa while LDH activity of epididymal tissue was not affected. In spite of the decrease in LDH per sperm cell when rats were exposed to constant light, in total epididymis (epididymis tissue plus sperm cells content) and in spermatozoa, values of enzyme activities expressed per weight unit were higher than those of controls. This is explained by the increase in the amount of stored spermatozoa, both in caput and cauda, produced by exposure of animals to constant light.

Our results confirm that in rats, chronic exposure to constant light promotes a reduction of fertilizing ability and indicates that continuous lighting reduces the total LDH and LDHC₄ activities, possibly due to moderate aging of spermatozoa with-

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in the duct by lengthening of the sperm transit through the epididymis.

INTRODUCTION

Mammalian spermatozoa are known to undergo important morphological, physiological and biochemical modifications after leaving the testes and during their transit through the male reproductive tract (Bedford, 1990; Yanagimachi, 1994). During its passage through the epididymis, gamete undergoes a process of "maturation" leading to the acquisition of its fertilizing ability (Orgebin-Crist and Olson, 1984; Cooper, 1986; Robaire and Hermo, 1988). Some changes in the spermatozoa during maturation such as, protein content (Lavon *et al.*, 1971; Vermouth *et al.*, 1986), lipid content and composition (Aveldaño *et al.*, 1992), and enzyme activities (Vermouth *et al.*, 1986) were demonstrated in rat.

The epididymis displays regional variations in the morphology and metabolic properties of its epithelium (Hamilton, D.W., 1975), which are relevant for the progressive development of mature sperm characteristics. This duct has spontaneous peristaltic contractions and receives sympathetic innervation (Hib, 1976). The surgical removal of the inferior mesenteric ganglion and proximal hypogastric nerves produces significant changes in epididymal sperm transport, sperm motility and luminal fluid proteins composition (Ricker *et al.*, 1997). On the other hand, it is known that sympathetic neurotransmission is modulated by melatonin, a hormone synthesized and released by the pineal gland (Wurtman, 1967).

The hormonal activity of the pineal gland plays the role of internal timer for several daily and seasonal phenomena (Reiter, 1991; Hardeland and Rodriguez, 1995) and appears to exert an important role in neuroendocrine regulation. Melatonin apparently affects the reproductive function at the hypothalamic-pituitary level by inhibition of the hypothalamic pulsatile secretion of gonadotrophin releasing hormone. This also takes place at the gonadal level where melatonin receptors have been found. Moreover, maternal pineal influences the gonadal development and function of rat offspring (Jarrige and Boucher, 1992; Vermouth *et al.*, 1995).

Although the rat is a non-seasonal breeder, its reproductive function is influenced by the photoperiod, melatonin administration or pinealectomy. It has been demonstrated that melatonin synthesis is suppressed by constant lighting (Moore and Klein, 1974; Reiter, 1981). Light acts as a selective inhibitor of hydroxyindole-o-methyl transferase (HIOMT), which is required for melatonin synthesis (Wurtman *et al.*, 1963). Earlier work conducted by us in rat testis (Vermouth *et al.*, 1984) have shown that constant light disrupts the circadian variation of lactate dehydrogenase isozyme C₄ (LDHC₄), a sperm-specific isozyme (Blanco *et al.*, 1975; Blanco, 1991). Moreover, Carneiro *et al.* (1991) showed in the rat that the circadian rhythm of vas deferens contraction is abolished when melatonin synthesis is suppressed by constant lighting.

One question arising from these studies regards whether melatonin suppression influences the epididymis and the spermatozoa enzymatic activity. In the present study we have suppressed rat melatonin synthesis by constant light. Lactate dehydrogenase (LDH: EC 1.1.1.27) and protein content were analyzed in whole epididymis, epididymal tissue separated from spermatozoa and in sperm cells of rats chronically exposed to constant light. We show that constant light induced a reduction of total LDH and LDHC₄ per epididymal spermatozoa while total LDH activity of epididymal tissue was not affected.

MATERIALS AND METHODS

Animals

Sexually rested male Wistar rats were kept in a temperature-controlled room ($23 \pm 2^\circ\text{C}$), housed in groups of five in cages with water and food (standard lab chow) "ad libitum". Control rats were kept in a light-dark cycle of 14 h light and 10 h of darkness. The lights on phase was from 06:00 to 20:00 h. During the light period, light intensity was 150-200 lux at the level of the cages. These animals were designated as group L:D. The experimental group was composed by animals maintained from birth in the same conditions as controls, but exposed from the age of 60 days to constant light (L:L) for 50 days. Approximately one-half of the rats of each group was used to test the fertilizing ability.

Preparation of extracts

Animals were sacrificed by decapitation at 10:00 h because the lowest level of enzymatic LDH rhythm was observed at this time (unpublished results).

Epididymis were removed and carefully dissected to eliminate adventitious tissue. Each epididymis was sectioned into three segments: proximal (caput), medial (corpus) and distal (cauda). Corpus was discarded. Segments (caput and cauda) of one epididymis from each animal were cut into pieces of approximately 1 mm³ and suspended in a pH 7.4 solution containing 115 mM NaCl, 5 mM KCl, 3 mM MgCl₂·6H₂O, 15 mM NaH₂PO₄·H₂O, 1 mM ethylenediamine tetraacetic (EDTA) and 20mM Tris. After gentle stirring at 30°C for 5-6 min, the suspension was filtered through a silk cloth. The debris retained in the cloth was washed four times with the same solution in order to recover as many spermatozoa as possible. The filtrates were centrifuged at 800 g for 10 min at room temperature. The low centrifugation speed maintained spermatozoa integrity. The absolute quantity of spermatozoa content in caput or cauda epididymis is a critical value because it is subject to loss in the extraction procedure.

The pellet of spermatozoa was washed with 10 parts of the solution, centrifuged as indicated above and finally resuspended in 5 ml of buffer solution. An aliquot of this suspension, appropriately diluted with 0.15 M NaCl in 0.2% formaldehyde, was used to count spermatozoa in a haemocytometer in triplicate. The remaining fraction of sperm suspension was centrifuged at 13,000 g for 15 min at 4°C. The pellet was weighed and resuspended to obtain a final concentration of 10-20 x 10⁶ cells per ml. The sperm suspension was homogenized by ultrasonic treatment on ice (four 70 W pulses of 15 sec each at 10 sec intervals, in a Branson Sonic Power Co. Sonifier B-12). Total disruption of cells was checked by light microscopy. The sperm suspension were centrifuged at 13,000 g for 30 min at 4°C and supernatants were used for the studies.

The epididymal tissue remaining in the cloth was repeatedly rinsed with the pH 7.4 solution until it was freed of spermatozoa. The washed tissue was weighed and suspended in 9 parts of distilled water (1:10, W:V) and homogenized. The suspension was centrifuged at 13,000 g for 30 min at 4°C and the supernatant was used for the studies.

Segments (caput and cauda) of the opposite epididymis from each animal with their spermatozoa content were cut into pieces as described previously and followed the same procedure as indicated for the sections free of germ cells. Extracts from epididymis including the spermatozoa will be designated as "total epididymis" and those from washed epididymis without spermatozoa as "epididymal tissue".

Enzyme assays

Total lactate dehydrogenase was determined by the method of Wroblewski and La Due (1955), using 0.5 mM sodium pyruvate as substrate; lactate dehydrogenase isozyme C₄ was assayed with the same method by using 5.0 mM α -keto-glutarate as substrate. This substrate allows a selective determination of rat LDHC₄ in mixtures containing other molecular forms of lactate dehydrogenase (Shatz and Segal, 1969; Coronel *et al.*, 1983). The assays were performed at 37°C. Change of absorbance at 340 nm was measured for 3 min. One unit of enzyme is the amount producing a ΔE_{340} of 2.07 per min, which corresponds to the oxidation of 1 μ mole of NADH.

Protein determination

Soluble protein concentration was determined with the method of Lowry *et al.* (1951), using bovine serum albumin (Frac. V) as standard.

Control of spermatozoa contamination in the epididymis tissue

Total LDH and LDHC₄ activities were determined in epididymal tissue. Relative activity of pure rat LDHC₄ with different substrates has been determined in our laboratory (Coronel *et al.*, 1983). The ratio of activity assayed with 0.5 mM pyruvate over that determined with 5.0 mM α -ketoglutarate is 4.12 for rat LDHC₄. Thus, knowing the activity of a given amount of LDHC₄ with 5.0 mM α -ketoglutarate, it is possible to estimate that to be obtained if the assay were performed with 0.5 mM pyruvate as substrate. By relating this calculated LDHC₄ activity with total lactate dehydrogenase (0.5 mM pyruvate), an estimation of enzyme contamination from spermatozoa can be made in epididymal tissue. The percentage of LDH activity belonging to spermatozoa contamination in all experiments described gave a value lower than 5%.

Fertilizing ability test

To test the fertility, male rats maintained in L:D cycle or L:L were individually placed in separate cages with a soliciting estrous female for 24 hours. After this period, females were caged individually. In each case, the test was considered positive when the female became pregnant.

Statistical analysis

Student's t test was used to estimate significance of differences between two mean values; statistical significance was set at $P < 0.05$.

RESULTS

Mammalian spermatozoa volume changes while passing through the epididymal duct (Laufer *et al.*, 1979). Thus, to compare the protein content and the enzyme activity between the germ cells from caput and cauda segments, we prefer to express the results per 10^9 spermatozoa or per gram of gamete.

Soluble protein concentration

Epididymis

Constant light exposure over 50 days produced a significant protein concentration increase in caput ($P < 0.05$) and cauda ($P < 0.001$) of total epididymis (Fig. 1A). On the contrary, in epididymal tissue the protein concentration fell in both sections compared to that of animals in L:D ($P < 0.05$) (Fig. 1B).

Moreover, in both groups (L:D and L:L) the protein concentration per gram of wet tissue in caput was similar to that of cauda, either in total epididymis (Fig. 1A) or in epididymis free of spermatozoa (epididymal tissue) (Fig. 1B).

Spermatozoa

In the L:L group, the protein content per gamete expressed as $\text{mg} \times 10^9$ spermatozoa fell about 60% from caput to cauda ($P < 0.001$) (Fig. 1C) in a similar way to that of the group L:D.

On the other hand, when results were expressed as mg/gram of cells, the constant light induced an increment of protein content in spermatozoa of both epididymal segments ($P < 0.001$) compared with those from control animals (Fig. 1D). In groups L:D and L:L, the protein values in spermatozoa of caput were similar to those in spermatozoa from cauda.

Enzyme activity

Epididymis

In animals maintained in constant light, the LDH activity showed an increment in caput ($P < 0.001$) and cauda ($P < 0.05$) of total epididymis (Fig. 2A). Also, LDH activity in total epididymis (Fig. 2A) and epididymal tissue (Fig. 2B) showed higher values in cauda than in caput ($P < 0.001$) in both animal groups.

Spermatozoa

When enzymatic activity was expressed in Units per 10^9 spermatozoa, animals maintained in constant light showed a significant reduction of total LDH ($P < 0.01$) and LDHC₄ ($P < 0.001$) in spermatozoa from caput and cauda (Fig. 2C and 3A, respectively) compared with control group. Gametes from caput showed the highest value of total LDH ($P < 0.01$) and LDHC₄ ($P < 0.001$) activities (Fig. 2C and 3A, respectively). In the control group, the decrease of enzyme activities in the distal portion was 37% and 40% for total LDH and LDHC₄, respectively. In spermatozoa of group L:L, the total LDH and LDHC₄ activities fall 36% during its epididymal maturation from caput to cauda.

On the other hand, when the results were expressed as units/gram of cells, constant light induced an increment of total LDH and LDHC₄ activities in spermatozoa from caput and cauda epididymis (Fig. 2D and 3B). Also, in groups L:D and L:L, total LDH and LDHC₄ activities in cauda spermatozoa were higher than those in caput ($P < 0.001$ and $P < 0.05$, respectively).

By calculating the ratio between enzymatic activity values, expressed as units/ 10^9 spermatozoa and units/gram spermatozoa, it is possible to know approximately the spermatozoa mass ($\text{g}/10^9$ cells) in each epididymal segment. Both groups showed that the mass is significantly higher in caput than in cauda ($P < 0.05$). However, spermatozoa mass values in group L:L (caput: 0.56 ± 0.06 ; cauda: 0.24 ± 0.05) were notably lower than those of group L:D (caput: 0.98 ± 0.05 and cauda: 0.55 ± 0.07). These results showed a reduction of 47% and 56% in caput and cauda segments, respectively ($P < 0.01$).

Fertilizing ability

Results of fertility tests are summarized in Table I. The percentage of pregnancies in females mated

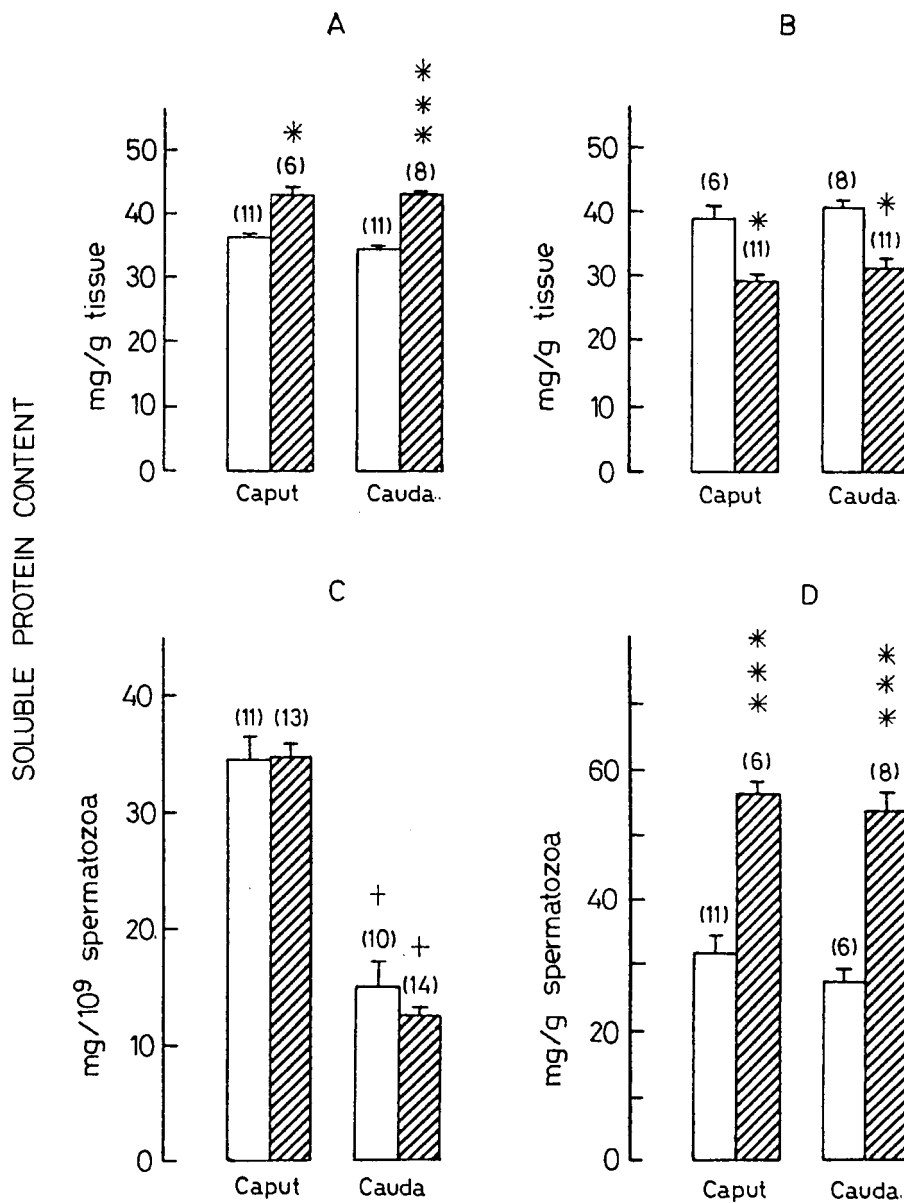


Fig. 1 - Soluble protein concentration in total epididymis (A), epididymal tissue (B), and spermatozoa (C and D), from caput and cauda. White bars indicate average value for animals maintained in a photoperiod of 14 h light: 10 h dark (L:D). Shaded bars correspond to animals maintained in constant light (L:L). Vertical lines on bars indicate standard error. Number of animals are shown in brackets. + indicates $P < 0.001$ caput vs. cauda in comparison in the same lighting conditions. * $P < 0.05$ and *** $P < 0.001$ L:D vs. L:L in comparison for the same segment.

with males maintained in L:L was remarkably lower than those in females mated with males under L:D photoperiod (44% and 88% respectively).

DISCUSSION

It is clear that mammalian spermatozoa undergo an essential and rather complicated process of maturation as they pass through the epididymis. Once maturation is complete, spermatozoa are maintained in the cauda epididymides before being

expelled as a result of ejaculation into the ejaculatory duct. In the epididymis, sperm maturation occurs during storage prior to ejaculation. It is reasonable to assume that the time spent by spermatozoa in passing along the epididymal duct is related to these events. In the present study, constant light exposure, affected the fecundity of the male rats after natural mating, since their breeding rate was lower than those of untreated animals. Similar results have been reported by Fantie *et al.* (1984), who described a sexual behavior disrupt-

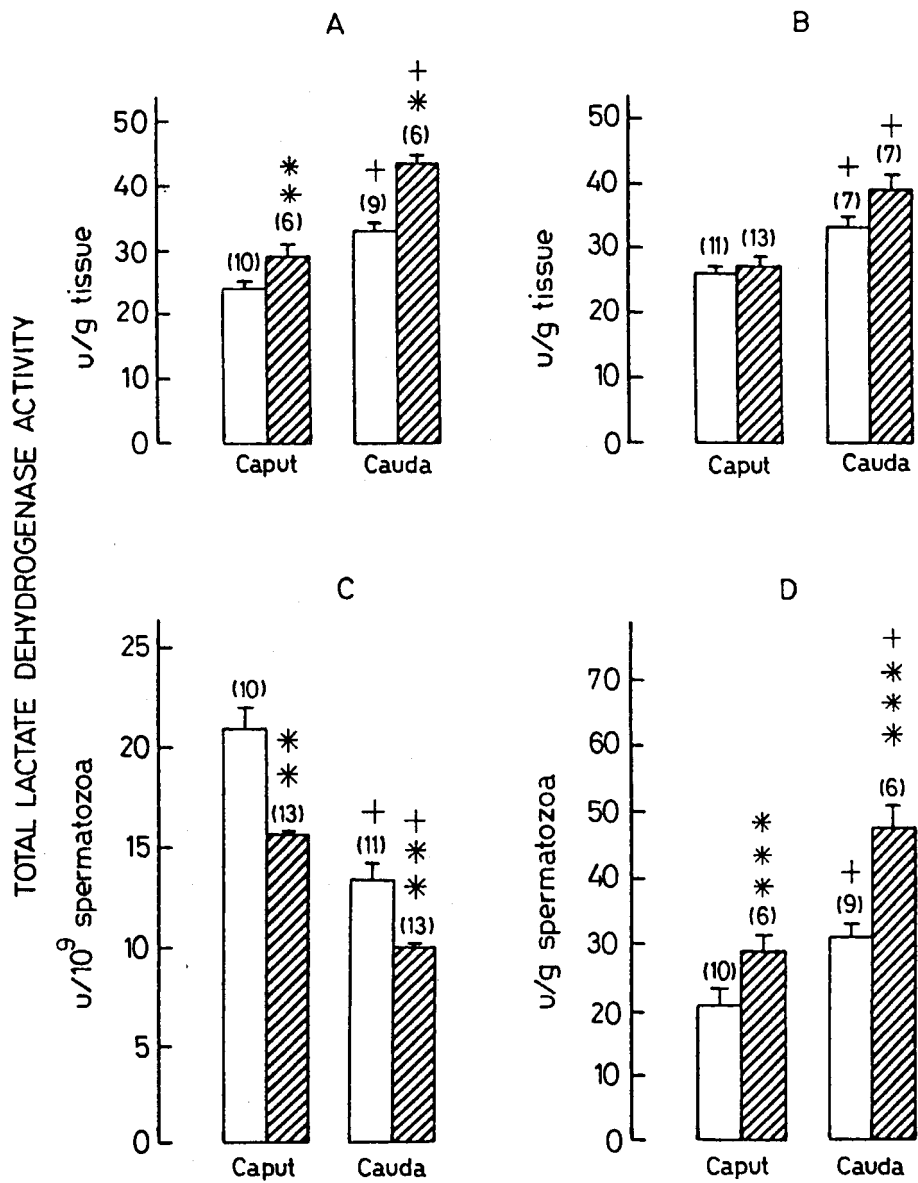


Fig. 2 - Total lactate dehydrogenase activity in total epididymis (A), epididymal tissue (B), and spermatozoa (C and D), from caput and cauda. White bars indicate average value for animals maintained in a photoperiod of 14 h light: 10 h dark (L:D). Shaded bars correspond to animals maintained in constant light (L:L). Vertical lines on bars indicate standard error. Number of animals are shown in brackets. + indicates $P < 0.001$ caput vs. cauda in comparisons. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ L:D vs. L:L in comparisons.

tion in animals exposed to constant white light for 60-90 days. Disruption of hormonal and behavioral rhythms could cause a deficit in rat ejaculation by disorganizing general activity rhythms. However, the mechanisms underlying these changes are not understood.

Spontaneous epididymal contractility is a factor that enables sperm migration, while transit time is determined by the frequency of contractions (Cosentino and Cockett, 1986; Robaire and Hermo, 1988). This duct receives sympathetic innervation from the inferior mesenteric ganglion and its surgi-

cal removal produces significant changes in epididymal sperm transport, sperm motility and luminal fluid protein composition (Ricker *et al.*, 1997). In addition, surgical sympathectomy increases the transit time and the number of spermatozoa in the cauda epididymis (Kempinas *et al.*, 1998b). On the other hand, in the prostatic portion of rat vas deferens, melatonin potentiates sympathetic neurotransmission by increasing contractions in response to noradrenaline and ATP released by acetylcholine stimulation of presynaptic nicotinic receptors (Carneiro *et al.*, 1994). The impairing

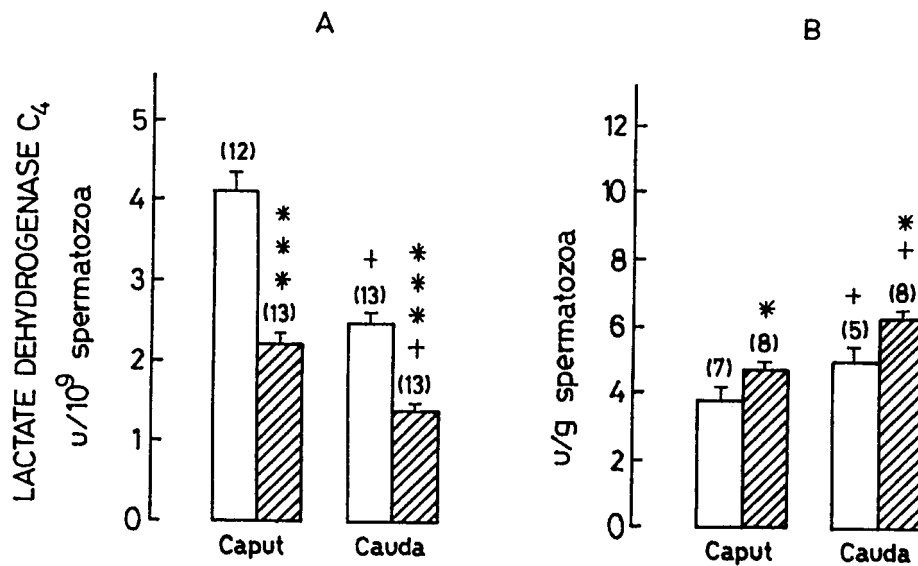


Fig. 3 - Lactate dehydrogenase isozyme C₄ activity in spermatozoa (A and B) from caput and cauda. White bars indicate average value for animals maintained in a photoperiod of 14 h light: 10 h dark (L:D). Shaded bars correspond to animals maintained in constant light (L:L). Vertical lines on bars indicate standard error. Number of animals are shown in brackets. + indicates P<0.001 caput vs. cauda in comparisons. *P<0.05, ***P<0.001 L:D vs. L:L in comparisons.

pineal nocturnal secretion of melatonin by constant light abolishes the daily variation in the maximal acetylcholine-induced contraction of vas deferens (Carneiro *et al.*, 1991, 1993). Thus, it seems that a suppression of melatonin secretion by constant light could reduce the epididymal contractions, and consequently, the transit time along the epididymis would be increased. This is detrimental for sperm maturation and results in the releasing of non-fertile spermatozoa into the ejaculate. Our experiments also demonstrated that in animals exposed to continuous illumination, the LDHC₄ activity per epididymal gamete decreased by 57% compared with controls. It is known that LDHC₄ is a component of shuttles that transfer reducing equivalents from cytosol to mitochondria (Burgos *et al.*, 1982; Coronel *et al.*, 1986) and play an important role when gametes utilize glycolytic substrates in aerobic metabolism. Therefore, a decrease of enzymatic activity per individual gamete induced by constant light could be a consequence of delayed sperm transport through the epididymal duct. The biochemical changes that sperm undergo during their epididymal transit enable them to fertilize a mature oocyte. Alternatively, the low fertility rates of light-treated males would be also attributable to sperm metabolic conditions. Whether the significant reduction in the spermatozoa specific enzyme activity implies changes in its fertilizing ability remains to be elucidated.

On the other hand, there were no effects on protein content of spermatozoa from epididymal segments of rats treated with constant light. However, there was a decrease of protein concentration in epididymal tissue free of spermatozoa in animals exposed to constant light (Fig. 1B). Relatively little is known about the effects of melatonin in this tissue, but there is some evidence for a correlation between melatonin modulation of sympathetic neurotransmission and protein synthesis. In this regard, Carneiro *et al.* (1994) showed that melatonin decreases [³⁵S]methionine incorporation in the hypogastric ganglion-prostatic vas deferens preparation. This suggests that melatonin modulates the presynaptic nicotinic response and also controls the trophic neuronal influence on smooth muscle cells.

The protein content in epididymal tissue free of spermatozoa in group L:L (Fig. 1B) was lower than that in control animals, while protein content per gamete did not differ significantly from the levels of untreated rats (Fig. 1C). Therefore, we may assume that the increment of protein content in total epididymis of group L:L (Fig. 1A) could be explained by an increased number of spermatozoa. A similar finding was described after surgical removal of the rat inferior mesenteric ganglia (Billups *et al.*, 1991). The results of these authors were interpreted as an increase of sperm transit time and retention of gametes within the epididymis. Thus, our results in animals exposed to

Table I
Breeding performance of male rats in different lighting conditions

Group	Number of males	Number of females	Number of pregnancies
L:D	17	17	15 (88%)
L:L	18	18	8 (44%)

L:D: 14 h light:10 h darkness; L:L: constant light.

constant light are comparable to that observed after inferior mesenteric ganglia ablation. This suggests an accumulation of epididymal sperm as a result of a delayed sperm transport by reduction of the epididymal contraction.

Also, it may be considered that the enhanced sperm number in the epididymal duct from group L:L reflects an increase of spermatozoa production by the testis. Testosterone is one of the requirements to maintain normal spermatogenesis. Because plasma testosterone levels were not found to be significantly altered in rat by continuous illumination (Fantie *et al.*, 1984), daily spermatozoa production probably was not affected. Furthermore, rat testis daily spermatozoa production remained unchanged when guanethidine selectively blocked sympathetic noradrenergic neurons. This treatment also induced a significant increase in sperm number in cauda epididymis (Kempinas *et al.*, 1998a). In addition, in a previous study we have shown that there was no significant difference in LDHC₄ activity rat testis between groups L:D and L:L (Vermouth *et al.*, 1984). For instance, there are no antecedents to presume that constant light affects rat testis daily spermatozoa production.

On the other hand, in group L:L, the slowing down of sperm transit could be related to alterations in fluid secretion in the epididymal duct. In this respect, it is known that chemical-induced sympathectomy prevents fructose secretion in prostate and seminal vesicle of rats (Lamano *et al.*, 1990). Moreover, previous works in our laboratory have shown that chronic exposure of rats to constant light promotes an alteration in sympathetic parotid and submaxillary glands secretory response (Chiarenza *et al.*, 1989; Gallará and Bellavía, 1995). Whatever the effect of continuous lighting on the secretory activity of the epididymis it remains to be elucidated.

In addition, we found that by expressing the pro-

tein content and enzymatic activity as per gram of spermatozoa, both values showed a significant increase in group L:L, while the estimated spermatozoa mass of group L:L was lower than that of group L:D. These findings could be explained by an enhanced number of cells per gram of gametes. In this case, it is known that as spermatozoa mature through the epididymal duct, they undergo a cytoplasmic loss, and the cellular volume decreases while the density increases (Lindahl and Thungvist, 1965; Laufer *et al.*, 1979). Thus, our findings hardly suggest a moderate gamete size reduction by aging phenomena in spermatozoa of the epididymal duct. These results are in agreement with data reported by Cuasnicu and Bedford (1989) demonstrating, in hamster, a closer correlation between restrained aging sperm and a reduced ability to fertilize an oocyte. This leaves us with the intriguing question whether periodic melatonin treatment would restore the gamete transit through epididymis and, as a consequence, fertilizing ability. Our results would appear to be of biological significance for human reproduction, with changes in the melatonin level following night-shift work, physical stress and sleep disorders (Monteleone *et al.*, 1992; Dawson and Encel, 1993; Arendt *et al.*, 1997).

In conclusion, the present study provides yet another piece of evidence that chronic exposure to constant light promotes a low fertilization performance, presumably by decreasing epididymal contractility. Thus, slowing down gamete transit through epididymis would be accomplished by a reduction of spermatozoa LDH activity possibly due to a moderate aging of spermatozoa within the duct. Whether the significant caput and cauda reduction in the sperm specific enzyme LDHC₄ activity implies changes in the fertilizing ability of spermatozoa remains to be elucidated.

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