Carnitine content of rabbit epididymal spermatozoa in organ culture

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Summary. Tubules of the rabbit proximal corpus epididymidis were maintained for 24 h *in vitro*. The carnitine content in cells from tubules cultured with dihydrotestosterone was greater than that in cells from tubules cultured without androgen. The effect of dihydrotestosterone was abolished by the simultaneous addition of cyproterone acetate to the medium. Culture of segments of the distal corpus for 24 h with dihydrotestosterone did not change the carnitine content of spermatozoa. These results support our hypothesis that the accumulation of carnitine is involved in sperm maturation.

Introduction

Carnitine is present in exceedingly high concentration in the epididymis of rats (Marquis & Fritz, 1965a; Casillas, 1972; Brooks, Hamilton & Mallek, 1974) and other species (Casillas, 1972). Carnitine accumulates in bovine (Casillas, 1973) and in rabbit (Casillas & Chaipayungpan, 1979) spermatozoa as they mature in the epididymis and we have suggested that one of the functions of the organ is to supply carnitine to spermatozoa as they mature. We have also presented evidence that carnitine plays a special role in the metabolism of mature bovine and monkey spermatozoa (Casillas & Erickson, 1975a). In addition, many others have studied the occurrence of carnitine in the male reproductive tract and its relation to the metabolism of the epididymis and spermatozoa and these studies have been recently reviewed (Brooks, 1980; Hinton & Setchell, 1980; Carter, Stratman, Hutson & Lardy, 1980).

Marquis & Fritz (1965b) first demonstrated that testosterone was necessary to maintain epididymal carnitine levels in rats and Casillas & Erickson (1975b) confirmed these results and also showed that epididymal tissue and spermatozoa lack the ability to synthesize carnitine *de novo*. Although Casillas (1973) noted that bovine spermatozoa from the caput epididymidis accumulate isotopically-labelled carnitine *in vitro* while spermatozoa from the cauda epididymidis do not, the rate of accumulation was not increased by testosterone or dihydrotestosterone. Furthermore, the optimal rate of carnitine uptake *in vitro* was too slow to produce significant increases in the cellular carnitine content during the relatively short incubation periods necessary to retain cellular viability. Orgebin-Crist, Danzo & Davies (1975) have reviewed the numerous investigations that show that the development and maintenance of sperm fertilizing ability in the epididymis is regulated by androgens. Sperm maturation can be induced in isolated tubules from the proximal corpus epididymidis *in vitro* by addition of 5 α -androstane-3,17 β -diol (dihydrotestosterone) to the culture medium (Orgebin-Crist & Tichenor, 1973) and this induction can be suppressed by the further addition of cyproterone acetate (Orgebin-Crist & Jahad, 1978). We have now studied the uptake of carnitine by spermatozoa in isolated epididymal tubules *in vitro*.

Materials and Methods

Animals

Adult rabbits of proven fertility and of various breeds (primarily, New Zealand White) were obtained from a local rabbit breeder: 190 animals were used. Immediately after killing the animals, the testes and epididymides were removed from the scrotum under sterile conditions and the proximal corpus epididymidis was prepared for culture.

Culture methods

Tubules were prepared for culture by a slight modification of the procedure described by Orgebin-Crist & Jahad (1978). As much connective tissue as possible was removed without rupturing the tubule which was then placed in a 0.1% solution of collagenase for 30 min at 37°C to digest the remaining connective tissue. After enzyme treatment, the tubule was gently, but thoroughly, rinsed with culture medium and then placed in the centre of a Petri dish. The tubules were cultured in Medium 199 supplemented with 15% calf serum, a penicillin–streptomycin mixture (100 i.u.), and 25 mM-L-carnitine for 24 h at 31°C in an incubator that was continuously flushed with air containing 5% CO₂. Dihydrotestosterone and cyproterone acetate were dissolved in a small volume of ethanol and added to the appropriate culture medium with the final ethanol concentration less than 1%. Equal concentrations of ethanol were added to the appropriate control dishes.

Carnitine analysis of spermatozoa

After culture, the tubules were removed from the medium, lacerated in a small volume of buffered salt solution containing 20 mM-NaH₂PO₄, 119 mM-NaCl, 5 mM-KCl and 2 mM-MgCl₂ (pH 7.0). Cellular debris was removed from the suspension by filtration through cheese cloth and the spermatozoa were collected by centrifugation at 800 g for 5 min. The sperm-containing pellets were washed twice in 10 volumes of the buffered salt solution to remove the exogenous carnitine and the final pellet was suspended in 0.5 ml of the buffered salt solution. An aliquot of the suspension was removed and sperm counts were performed by conventional haemocytometer techniques. The rest of the suspension was analysed for carnitine as previously described (Casillas & Chaipayungpan, 1979). In brief, spermatozoa were suspended in 0.5 M-HClO₄ and were disrupted further by sonic irradiation. Acid-insoluble material was removed by centrifugation and the acid-soluble fraction was neutralized with KOH. The KClO₄ precipitate was removed by another centrifugation and the carnitine present in the clear, neutral extracts was determined by the method described by Cederblad & Lindstedt (1972) with modifications suggested by McGarry & Foster (1976). The limit of sensitivity was 5 pmol and the relative deviation of 5 duplicate analyses was 2–10%. In 3 experiments, the recovery of carnitine added to extracts of spermatozoa was 100 ± 10%.

Experimental design

In the first series of experiments, the proximal segments of each corpus epididymidis from 10 rabbits for each experiment were randomly assigned to a control group or to a group for culture with dihydrotestosterone. In the second series, the epididymal segments from 10 rabbits per experiment were assigned to groups for incubation with no additions (controls), dihydrotestosterone, cyproterone acetate or dihydrotestosterone plus cyproterone acetate. Another series of experiments was also performed, identical to those of Series 1 except that distal segments of the corpus epididymidis were used instead of proximal segments. Spermatozoa from each group were pooled before being analysed for carnitine. Differences between groups were compared by the *t* test.

Materials

Collagenase (Type II), dihydrotestosterone and carnitine acetyltransferase from pigeon muscles (98 units/mg protein) were obtained from Sigma Chemical Co., St Louis, Missouri; Medium 199 (Hanks BSS containing 1.4 g NaHCO_3/l), calf serum, and penicillin-streptomycin were from Microbiological Associates, Walkersville, Maryland; L-carnitine chloride was from Supelco Inc., Bellefonte, Pennsylvania; [acetyl- ^3H]acetyl Coenzyme A (sp. act. 1.97 Ci/mmol) was from New England Nuclear, Boston, Massachusetts; Dowex 2×8 (200–400 mesh) was from Biorad Laboratories, Richmond, California; sodium tetrathionate was from ICN Pharmaceuticals, Plainview, New York. Cyproterone acetate was a gift from Dr John Resko, Oregon Regional Primate Research Center, Beaverton, Oregon. All other reagents were of the best grade available from commercial sources.

Results

The carnitine content of spermatozoa from tubules maintained in culture with 0.5 $\mu\text{g}/\text{ml}$ dihydrotestosterone (8.3 ± 1.4 nmol/ 10^8 cells) was significantly ($P < 0.01$) greater than in spermatozoa maintained in the absence of androgen (3.1 ± 0.3 nmol/ 10^8 cells). These results are the mean $\pm$ s.e.m. for 15 separate experiments, 10 rabbits/experiment. The enhancement induced by dihydrotestosterone was suppressed by the simultaneous addition of cyproterone acetate to the culture medium (Table 1).

Table 1. Effect of cyproterone acetate on dihydrotestosterone (DHT)-stimulated carnitine uptake by spermatozoa

	Culture conditions			
	Control	DHT (0.5 $\mu\text{g}/\text{ml}$)	Cyproterone acetate (5 $\mu\text{g}/\text{ml}$)	DHT (0.5 $\mu\text{g}/\text{ml}$) + cyproterone acetate (5 $\mu\text{g}/\text{ml}$)
Carnitine content (nmol/ 10^8 spermatozoa)	$3.0 \pm 0.6^*$	$6.3 \pm 1.1^{*\dagger}$	2.0 ± 0.7	$3.8 \pm 0.5^\dagger$

Values are $\pm$ s.e.m. for 5 separate experiments, 10 rabbits/experiment. Sperm numbers ranged from 1.0 – 5.8×10^6 cells/analysis.

* Significantly different ($P < 0.01$).

† Significantly different ($P < 0.05$).

In four separate experiments, each with 10 rabbits, tubules from the distal corpus epididymidis were cultured for 24 h in the presence and absence of dihydrotestosterone. The carnitine content of spermatozoa from cultures containing dihydrotestosterone (5.0 ± 1.1 nmol/ 10^8 cells) was the same as from control cultures (4.8 ± 0.8 nmol/ 10^8 cells).

Discussion

The present results support our hypothesis that the accumulation of carnitine is one aspect of sperm maturation in the rabbit (Casillas & Chaipayungpan, 1979). For the most part, we used the culture system described by Orgebin-Crist & Tichenor (1973) and Orgebin-Crist & Jahad (1978) who have demonstrated that spermatozoa maintained in culture tubules from the proximal corpus epididymidis develop the ability to fertilize ova only when androgen is added to the medium. We have shown that in this system, with the same concentration of

dihydrotestosterone, (1) the spermatozoa accumulate carnitine, (2) the effect is blocked by the simultaneous addition of the antiandrogen, cyproterone acetate, and (3) dihydrotestosterone treatment had no effect on the carnitine levels of spermatozoa maintained in cultured tubules from the distal corpus epididymidis. Spermatozoa from this region of the rabbit epididymis *in vivo* are fertile (Bedford, 1966; Orgebin-Crist, 1967). The one major difference between the culture conditions used in the present study and those used by Orgebin-Crist & Jahad (1978) was that we added 25 mM-L-carnitine to the culture medium. In preliminary experiments using media without added carnitine the spermatozoa in tubules cultured with dihydrotestosterone appeared to contain more carnitine than those in control incubations, but the differences were not significant. Carnitine was therefore added because some of the carnitine normally present in epididymal fluids leaked out of the tubules during the 24 h incubation period. For example, by the end of the incubation about 75% of the total carnitine was present in the medium and 25% was associated with the wet, fragile tubules in 2 experiments. The results reported here must also be interpreted cautiously in the light of results reported by Orgebin-Crist & Jahad (1977) who found that 23% of 359 rabbits contained fertile spermatozoa in the proximal corpus epididymidis. It is possible, but not probable, that the tubules assigned to the test groups here contained more fertile spermatozoa than those assigned to the control group. Another indication that our present *in-vitro* studies approximate conditions found *in vivo* is the observation that the sperm carnitine values obtained are very similar to those found in spermatozoa removed from the corpus epididymidis of freshly killed rabbits (Casillas & Chaipayungpan, 1979). Finally, the present report extends the results of previous investigations that have shown that epididymal carnitine levels are under androgenic control (Marquis & Fritz, 1965b; Casillas & Erickson, 1975b) and suggest that the uptake of carnitine by spermatozoa during epididymal transit is also controlled by androgens.

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