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Role of ascorbic acid in metabolism of rat testis and epididymis in relation to the onset of puberty

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Abstract. The metabolism of ascorbic acid, cholesterol, serum testosterone level and activities of 3β and 17β hydroxysteroid dehydrogenases were studied in testis and Cauda epididymis of prepubertal, pubertal and postpubertal (5, 15, 30, 45, 55 and 60 day old) rats. The data showed that serum testosterone levels and 3β and 17β hydroxysteroid dehydrogenases were increased with the age. The ascorbic acid metabolism was found to be stabilized in testis at day 30 being comparable with the adult, whereas a spurt in its metabolism occurred by day 45 and a significant depletion in ascorbic acid content in relation to the passage of the first wave of spermatozoa through cauda epididymis. The results of this study clearly elucidate that ascorbic acid is involved in metabolism of testis and epididymis in developing postnatal rats, in relation to the increasing demands for attaining a stable hormonal milieu, and the onset of puberty and the passage of the first wave of spermatozoa, *via* the formation of its free radical monodehydroascorbic acid and charge transfer complex mechanism.

Keywords. Ascorbate metabolism; steroidogenesis; rat testis; epididymis.

Introduction

Ascorbic acid (AA) is a biologically active reductant whose metabolic significance in animal, human tissue and biological fluids has been extensively elucidated (Chinoy, 1978; Chinoy *et al*., 1982). Its involvement during steroidogenesis is *via* the formation of its free radical, monodehydroascorbic acid (MDHA) (Agrawal and Laloraya, 1977; Chinoy *et al*., 1978) which if coupled with steroids, *viz*., pregnenolone and testosterone might produce progesterone or their active metabolites in the rat corpora lutea or testis. That AA influences the activity of 3β hydroxysteroid dehydrogenase in normal toad testis and synergizes with luteinizing hormone in hypophysectomized toad testis *in vitro* has also been suggested (Biswas, 1971). The combined role of trophic hormones, C-AMP, prostaglandins and AA in the regulation of adrenal and gonadal steroidogenesis, each acting at various levels in hypothalamo-hypophyseal-adrenal/gonadal axis is reported (Datta and Sanyal, 1978). An interrelationship exists between AA and sex hormones in AA synthesizing tissues and steroidogenic organs of male and female rats (Chinoy and Rao, 1979; Chinoy *et al*., 1979; Chinoy and Seethalakshmi, 1978).

Abbreviations used: AA, Ascorbic acid; MDHA, monodehydroascorbic acid; ASG, ascorbigen; AAU, AA utilization; AA-MM, AA macromolecule; AA-FR, AA free radical; TAA, total AA; DHA, dehydroascorbic acid; RAA, reduced AA; GSH, glutathione; HSD, hydroxysteroid dehydrogenase.

However, the ascorbate metabolism in testis and epididymis in relation to the onset of puberty and the possible role of AA and its mechanism of action during steroidogenesis in gonad is not yet fully understood. Hence, the present study was undertaken in postnatal rats ranging in age from 5-65 days.

Materials and methods

Healthy male albino rats (*Rattus norvegicus*) of Holtzman strain of different age groups (5, 15, 30, 45, 55 and 65 day old) were used for the experiments. They were maintained on a standard diet (Hindustan Lever Ltd., Bombay) and water was provided *ad libitum.* The animals were caged in an air conditioned animal house at a temperature of 20°C and exposed to 12–14 day light hours. The animals of each group were sacrificed accordingly and their testis and cauda epididymis were used for biochemical estimations. The testis and cauda epididymis were excised, blotted free of blood and were weighed on a torsion balance to the nearest milligram.

Ascorbic acid

The levels of free ascorbate (free AA), ascorbigen (ASG) AA utilization (AAU) and AA macromolecule (AA-MM) complex were carried out according to the method of Chinoy *et al*. (1976) and expressed as mg/g fresh tissue wt.

Ascorbic acid free radical (AA-FR) forming special peroxidase activity was assayed by the method of Chinoy (1973). The units were activity/g fresh tissue wt/20 min.

Total AA (TAA), dehydroascorbic acid (DHA) and reduced AA (RAA) were estimated by the method of Roe and Kuether (1943). The concentrations were expressed as μg/100 mg fresh tissue wt.

Glutathione (GSH) levels were estimated by the modified method of Grunert and Phillips (1951) and expressed as μg/100 mg fresh tissue wt.

The total cholesterol was assayed in testis and epididymis by the method of Pearson *et al*. (1953) and the concentrations were expressed as mg/100 mg fresh tissue wt. The histochemical localization of 3β and 17β hydroxysteroid dehydrogenases (HSDs) were carried out by the method of Tso and Lofts (1977).

In vitro study on sliced testicular tissue was done by the method of Dorrington and Fritz (1975). The testicular slices (200 mg) from post-pubertal rats were incubated with 4 ml of Kreb's Ringer bicarbonate buffer with 0·1% glucose (KRBG, pH 7·4). Pregnenolone (500 μg/ml) was added to the medium with or without supplementation of 0·001M AA (176 μg/ml).The tissue slices were incubated for 2 h at 31°C in a continuous atmosphere of 95% O_2 and 5% CO_2 until analyzed for testosterone levels.

Testosterone levels by RIA

Blood of different age groups of rats were collected by cardiac puncture at 11.30 in the morning in every case so as to avoid diurnal fluctuations in hormonal levels. The testosterone levels were determined in serum/medium by the method of Castro *et al*. (1974) at the Institute for Research in Reproduction, Bombay. The antiserum was supplied by the World Health Organization. It was raised in sheep against testosterone-

3-(O-carboxymethyl) Oxime-bovine serum albumin. It cross-reacted with DHT to 14% without any significant reaction with other steroids. The sensitivity of the assay was 3–5 pg/tube. The intraassay and interassay coefficients of variation were 6 and 7% respectively and the levels were expressed as ng/ml medium/serum.

For all quantitative studies, a minimum of six replicates were done and their results were statistically analyzed using student's '*t*' test.

Results

Six groups of rats ranging in age from 5-65 days were used. Each group contained 15-30 animals.

Ascorbate turnover pattern

The levels of free AA were significantly ($P < 0{\cdot}001$) higher in testis of 45 day old rats followed by 15 and 55 day old ones. In 30 and 65 day old rats, the levels were more or less the same and least in 5 day old rats. In cauda epididymis, highest AA was found in 45 day old rat followed by 30 and 55 and least was in 65 day old rats (table 1).

The bound ASG was not detectable in testis of 45 and 55 day old rats, whereas in 65 day old ones, it was significantly ($P < 0{\cdot}001$) more than in the other three age groups. In cauda epididymis, the ASG levels were declined progressively with age from 45–65 day old rats. In 25 day old ones, it was not detectable (table 1).

The rate of AAU was high and almost the same in 5 and 15 day old rats. It progressively enhanced from day 30 onwards to 55 day old rat testis and subsequently declined abruptly in 65 day old rat to the level found in 30 day old ones. In cauda

Table 1. Showing the levels of free AA, ASG, AAU, AA-MM complexing and the activity of AA-FR forming special peroxidase in testis and cauda epididymis of pubertal (5, 15, 30 day old), pubertal (45 and 55 day old) and postpuberal (65 day old) rats.

		Age in days					
Parameter	Tissue	5	15	30	45	55	65
Free AA*	Testis	1·16±0·13	5·89±0·9	3·2±0·1	7·6±0·3	4·12±0·2	3·0±0·2
	Cauda	—	—	14·2±1·2	19·5±0·8	11·4±0·7	6·03±0·01
ASG*	Testis	0·42±0·04	0·43±0·07	0·33±0·1	ND	ND	0·79±0·12
	Cauda	—	—	ND	5·8±0·4	2·32±0·5	0·91±0·07
AAU*	Testis	45±0·9	53±2	18±0·3	31·5±1·6	49·6±3·2	17·8±2·3
	Cauda	—	—	37±2·1	46·5±1·3	35·5±3·8	22·6±1·3
AA-MM*	Testis	ND	ND	ND	ND	ND	1·39±0·4
	Cauda	—	—	ND	ND	ND	4·21±0·5
AA-FR**	Testis	28±2·6	6·14±0·35	3·87±0·2	8·12±0·1	14·8±0·8	3·4±0·2
	Cauda	—	—	9·3±0·8	13·2±0·3	11·6±0·6	18·6±0·2

Values are mean ±S.E.
* mg/gm fresh tissue weight.
** Activity/gm fresh tissue weight.
ND = Not detectable.

epididymis the highest AAU was obtained in 45 day old rats, followed by 30 and 55 day old ones. The least was observed in cauda epididymis of 65 day old group (table 1).

The net bound complexing of AA (AA-MM complex) was not detectable in both testis and cauda epididymis of any age group rats, except in the 65 day old ones. The AA-FR peroxidase activity in the testis showed the same trend as AAU, whereas in cauda epididymis, the enzyme activity increased in 45, 55 and 65 day old rats than in 25 day old animals. The latter (65 day old rats) had the highest activity (table 1).

TAA, DHA, RAA and GSH

The levels of TAA were less in the testis of 5-55 day old rats as compared to 65 day old ones, whereas, in cauda epididymis the levels were higher in 30 and 65 day old rats than in 45 and 55 day old ones which had comparable values. The DHA decreased in 5-55 day old rat testis and was significantly ($P < 0{\cdot}01$) higher in 65 day old rats as compared to others. In epididymis, DHA increased in 55 and 65 day old rats in comparison to 30 day prepubertal ones, but was least in 45 day old rats. RAA was not detectable in the testis of prepubertal and pubertal rats except 30 day old ones, but increased in 65 day old rats. In epididymis, a decrease at 45 days and nondetectable levels at 55 days were observed, but the highest was again in 65 day old rats (table 2).

The GSH levels were increased throughout in 45, 55 and 65 day old rat testis than 5 and 30 day old groups. In cauda epididymis, it declined at 45 and 55 days and then increased in 65 day old rats (table 2).

Cholesterol

The cholesterol levels in the testis and epididymis were found to be higher in pubertal rats (45 day old rats) and subsequently a marked depletion occurred ($P < 0{\cdot}001$) at 65 days. In 5 and 15 day old rat testis, the cholesterol was significantly ($P < 0{\cdot}001$) lower in comparison to other groups (table 2).

Table 2. Showing the contents of TAA, DHA, RAA, GSH and cholesterol in testis and cauda epididymis of different age groups of rats.

		Age in days					
Parameter	Tissue	5	15	30	45	55	65
TAA*	Testis	0·11 ± 0·01	0·09 ± 0·01	0·11 ± 0·01	0·09 ± 0·01	0·08 ± 0·001	0·74 ± 0·1
	Cauda	—	—	0·72 ± 0·13	0·52 ± 0·16	0·59 ± 0·16	0·76 ± 0·08
DHA*	Testis	0·26 ± 0·01	0·11 ± 0·01	0·07 ± 0·01	0·12 ± 0·01	0·11 ± 0·01	0·39 ± 0·07
	Cauda	—	—	0·39 ± 0·05	0·28 ± 0·04	0·62 ± 0·04	0·42 ± 0·08
RAA*	Testis	ND	ND	0·04 ± 0·001	ND	ND	0·38 ± 0·04
	Cauda	—	—	0·33 ± 0·05	0·24 ± 0·03	ND	0·38 ± 0·03
GSH**	Testis	12·5 ± 0·72	15·2 ± 1·5	13·2 ± 0·58	17·7 ± 0·58	16·5 ± 1·02	16·2 ± 1·38
	Cauda	—	—	32·6 ± 3·26	24·5 ± 1·50	29·3 ± 3·1	47·8 ± 6·14
Choles-	Testis	0·14 ± 0·01	0·18 ± 0·002	0·25 ± 0·01	1·07 ± 0·01	0·52 ± 0·08	0·32 ± 0·01
terol***	Cauda	ND	ND	0·30 ± 0·04	0·43 ± 0·02	0·36 ± 0·03	0·28 ± 0·02

Values are mean ± S.E. * mg/gm fresh tissue weight. ** μg/100 mg fresh tissue weight. *** mg/100 mg fresh tissue weight. ND = not detectable.

Hormonal levels

Significantly ($P < 0{\cdot}02$; $P < 0{\cdot}001$) higher levels of circulating testosterone were found in adult rats as compared to those of other groups. The testosterone production *in vitro* by postpubertal rat testicular slices were found to be comparatively more ($P < 0{\cdot}5$) in the medium containing pregnenolone + ascorbic acid than the medium having pregnenolone alone (table 3).

The activities of 3β and 17β HSDs in rat testis were increased from pubertal to adult stage as is evident from the decreased duration of the incubation period and the increased intensity of the formazan granule deposition (table 3).

Table 3. Showing the circulating levels of testosterone, activities of 3β and 17β HSDs of testis in prepubertal, pubertal and postpubertal rats and influence of AA in rat testicular testosterone synthesis *in vitro* using pregnenolone.

Group	Testosterone (ng/ml medium)		Testoterone (ng/ml serum)	3β/17β HSDs	
	Pre. alone	Pre. + AA		Incubation time (h)	Intensity
Prepubertal	—	—	0·22 ± 0·03 (6)	5	+ (less)
pubertal	—	—	0·60 ± 0·10 (6)	4-5	+ + (moderate)
Postpubertal	6·68 ± 0·5 (4)	8·02 ± 0·7* (4)	2·37 ± 0·74 (5)	3½	+ + + (highest)

Values are mean ± S.E. Pre. = Pregnenolone, + = Intensity of formazan granule deposition. The number in parenthesis indicates '*n*' value. * $P < 0{\cdot}5$

Discussion

The ascorbic acid metabolism in the 30 days old rat testis was found to be more or less stabilized since majority of the parameters involved were almost similar to the adult rats. This period in the postnatal development of the rat seems to be a crucial one involving rapid structural changes and metabolic activity in preparation for the onset of puberty. The observations of the present study are supported by those of ultra-structural changes in rat cauda epididymis and vas deferens (Chinoy and Asok Kumar, 1982; 1983) as well as those on localization and assay of AA in reproductive tissue of male rats (Chinoy *et al*., 1983 a, b; 1984).

The ascorbic acid levels showed a spurt on day 45 in both testis and epididymis with the onset of puberty, concomitant with a decline in the levels of bound ascorbic acid. It implies that around day 45 an active mobilization of the bound ascorbate to its free form occurs for its subsequent rapid utilization, which increased with age, reached maximum levels by day 45 and thereafter declined in both tissues of 65 day old rat. This might be due to the increased requirements of AA for the animal which is attaining reproductive maturation. In 55 day old animals with the passage of the first wave of spermatozoa through the epididymis, the bound ascorbic acid was also significantly

depleted (in epididymis) suggesting AAU by sperms for their active metabolism and motility in agreement with our earlier data (Chinoy and Buch, 1977; Chinoy, 1978; Chinoy *et al*., 1982).

That AA is involved in steroidogenesis of the gonads has been reported (Agrawal and Laloraya, 1977; Datta and Sanyal, 1977; Chinoy *et al*., 1982). The present data supports these findings. A significant increase in circulating testosterone levels in postpuberal rats was accompanied by alterations in cholesterol levels in testis and Leydig cell activity. Chinoy *et al*. (1983) reported that AA deficiency in guinea pigs caused an and rogen deprived effect to the target organs by probably influencing Leydig cell/or by affecting testosterone synthesis in testis. The scorbutic condition also reduced the fertilizing ability of guinea pigs and the contractility of vas deferens. These data reveal that AA is essential for the maintenance of the structural and the functional integrity of androgen target reproductive organs (Chinoy *et al*., 1983a, b; 1984). The primary action seems to be at the testicular level and the other changes were secondary manifestations as a consequence of the primary effect (Chinoy *et al*., 1984).

The 65 day postpubertal rat testis and cauda epididymis showed AA-MM complexing for the first time during post-natal development. Similarly the enzymatic AAU as evidenced by AA-FR peroxidase activity occurred in testis only on day 65 but was initiated earlier in the epididymis of 45 day old rats. The synthesis of TAA was more in 65 day old rat than in other age groups. It is known that the levels of ascorbic acid in testis and epididymis are significantly greater than in liver, where it is synthesized. Moreover, tissues possessing a higher rate of metabolic activity have a correspondingly higher turnover of AA (Chinoy, 1978; Chinoy *et al*., 1982) and a profound sex difference exists in its levels in rat tissues where the males possess higher concentration as compared to females.

It is evident from the present data that AA acts in testis and epididymis *via* the formation of its free radical MDHA, which is eventually fully oxidized to DHA. The MDHA by virtue of possessing an unpaired electron is a much more powerful reducing agent than AA itself and is known to be involved in several oxido-reduction reactions, enzyme activation and potentiates the anabolic action of steroid in rat testis and epididymis (Kutsky, 1973; Lewin, 1976; Chinoy, 1978; Chinoy *et al*., 1982) by forming charge transfer complexes with macromolecules (Chinoy *et al*., 1978). Therefore, the mechanism of action of ascorbic acid is *via* the paramagnetic electron flow from MDHA and charge transfer complexing with macromolecules during postnatal development in rats. MDHA is an additional source of electron energy together with the energy obtained through the conventional breakdown of ATP in corroboration with our earlier data (Chinoy *et al*., 1983 a, b; 1984).

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