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Morphological and quantitative analysis of prepubertal rat germ and Sertoli cells in conditions of vitamin A-deficiency

N. Atanassova, L. Kancheva

Institute of Cell Biology and Morphology, Bulgarian Academy of Sciences, Sofia

Control and vitamin A-deficient (VAD) rat testes were investigated on days 3, 6, 12, 20 and 25 after birth. The germ cell counting on day 3th p.p. showed nonsignificant differences between VAD and control testes. The quantitative study during prepubertal period demonstrated a reduction of germ cell number and their ^{3}H-Thymidine labelling index. In- and B-spermatogonia were considerably reduced whereas A-spermatogonia decreased in a lesser extent. The Sertoli cell number and their labelling index were unchanged. Ultrastructural observations revealed normal structure of Sertoli cells while germ cell showed varying degenerative features. The obtained results suggest that during prepubertal period when significant changes occur in testicular cell populations, VAD severely affected germ cell structure and proliferation.

Key words: vitamin A-deficiency, germ cells, Sertoli cells.

Introduction

Recently it has been established that in addition to the hormonal regulation (FSH, LH and testosterone) spermatogenic function is controlled by an intragonadal paracrine system. Interactions between germ, Sertoli, Leydig and peritubular cells have been demonstrated by many authors [14].

An approach in the investigation of paracrine regulation of spermatogenesis is selective in vivo alterations of seminiferous epithelium using various experimental models and the study of different parameters of principal testicular cell populations. Vitamin A-deficiency is a good model for the study of interactions between germ and Sertoli cells.

Vitamin A in the form of retionol has been known to be essential for normal male reproduction. A deficiency of this vitamin in adult rats results in a degeneration and loss of germ cells which leads to an arrest in spermatogenesis at preleptotene stage [7]. Quantitative studies on adult VAD rat testes by Mitranond et al. [12] showed a decrease of the number of different types germ cells, while Unni et al. [19] demonstrated a reduction of mitotic activity of spermatogonial cells. According to the recent autoradiographic studies [10, 12] there appears to be a mitotic division arrest of A-spermatogonia in adult VAD rat testes. This type spermato-

gonia is responsible to reinitiation of spermatogenesis in synchronized manner after vitamin A-replacement [4].

The ultrastructural study of above-mentioned authors showed different degrees of degeneration of germ and Sertoli cells and their intercellular contacts.

The exact mechanism by which vitamin A-deficiency acts on the process of spermatogenesis is still unknown. Whether this effect is direct on germ cells or mediated by Sertoli cells is under discussion. On the base of biochemical studies about retinol esterification and accumulation in Sertoli cells S h i n g l e t o n et al. [17] postulated a hypothesis that Sertoli cells are central place in retinol metabolism of seminiferous tubules. E s k i l d et al. [3] demonstrated maximal levels of mRNAs for cellular retinol binding protein (CRBP) in early postnatal period.

There is no information about the effect of VAD on male gametogenesis during prepubertal period when important and significant changes occur in testicular cell populations that precede the oncet of spermatogenesis and determine the fertility in the adults. The aim of present work was to investigate the quantitative and morphological changes in principal cell types of prepubertal rat testes in conditions of vitamin A-deficiency.

Materials and methods

Female Wistar rats were fed on diet without vitamin A (AIN-76™ Purified diet for rats and mice [1]) at first day of pregnancy (when spermatozoa were found in the vaginal smears in the morning). New-born male rats continued to receive the same diet. The water was given ad libitum. Control and VAD male rats were killed on days 3th, 6th, 12th, 20th and 25th after birth. For electron microscopy (EM) the testes were fixed in 2,5% glutar aldehyde, postfixed in 1% OsO_4 and embedded in Durcupan. EM observations and microphotos were made on Opton EM 109. The selective 6-day-old animals were injected i. p. with ^{3}H-Thymidine (1 μCi/g body weight) and were killed after 3 hours. The testes were fixed in Serra's fixative and embedded in paraffin. Deparaffined sections 5 μm were dipped in Ilford-K_2 emulsion and processed for autoradiography. The quantitative analysis on 3, 6, 12 and 20 day p. p. was made on semithin Methyleneblue-Azur II-basic Fuchine stained sections. The general germ and Sertoli cell number and the number of different types germ cells were established in circular cross-sections of seminiferous cords and tubules. The per cent of labelled germ and Sertoli cells was counted in autoradiograms. For statistical analysis Students's *t*-test was used.

Results

On day 3th p. p. the counting of germ cells per cross-section of a seminiferous cord showed nonsignificant differences between VAD and control testes. The quantitative study during prepubertal period (6, 12 and 20 day) displayed a rapid decrease in general germ cell number in VAD animals comparing with the controls (Fig. 1). It is obviously that the tendency of several times multiplication of normal germ cells number during prepubertal period was absent in VAD specimens where a slight increment of that parameter was established. The Sertoli cell number per cross-section of a seminiferous cord and tubule during investigated period showed nonsignificant differences between VAD and control rats (Fig. 2).

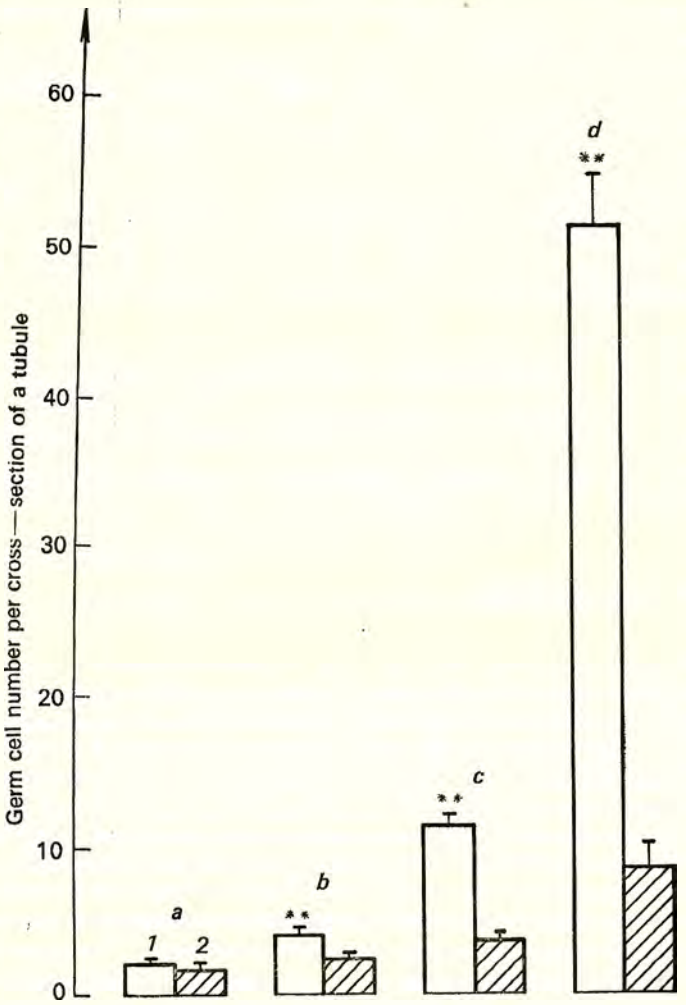

Fig. 1. Germ cell number per cross-section of a seminiferous cord and tubule of 3- (*a*), 6- (*b*), 12- (*c*) and 20- (*d*) day-old control (*1*) and VAD (*2*) rat testes. Data represent the mean $\pm$SD; ** — $p<0{,}01$

Autoradiographic data on day 6th p. p. demonstrated 25 % decrease of ^{3}H-Thymidine germ cell labelling index in VAD testes. The differences of Sertoli cell labelling index in VAD and control specimens was not significant (Fig. 3).

The quantitative study of different types spermatogonia on day 12th showed a reduction of relative number of differentiated spermatogonia (A-, In- and B-sg) in VAD rats whereas the number of undifferentiated spermatogonia increased (Fig. 4). At this age only in VAD animals a considerable number of degenerating germ cells in division was established.

In 20-day-old control and VAD testes meiotic germ cells were present but the most advanced in spermatogenic cell differentiation were the pachytene spermatocytes. The seminiferous tubules of VAD testes contain Sertoli cells, spermatogonia and few spermatocytes. The relation of spermatocyte to spermatogonial

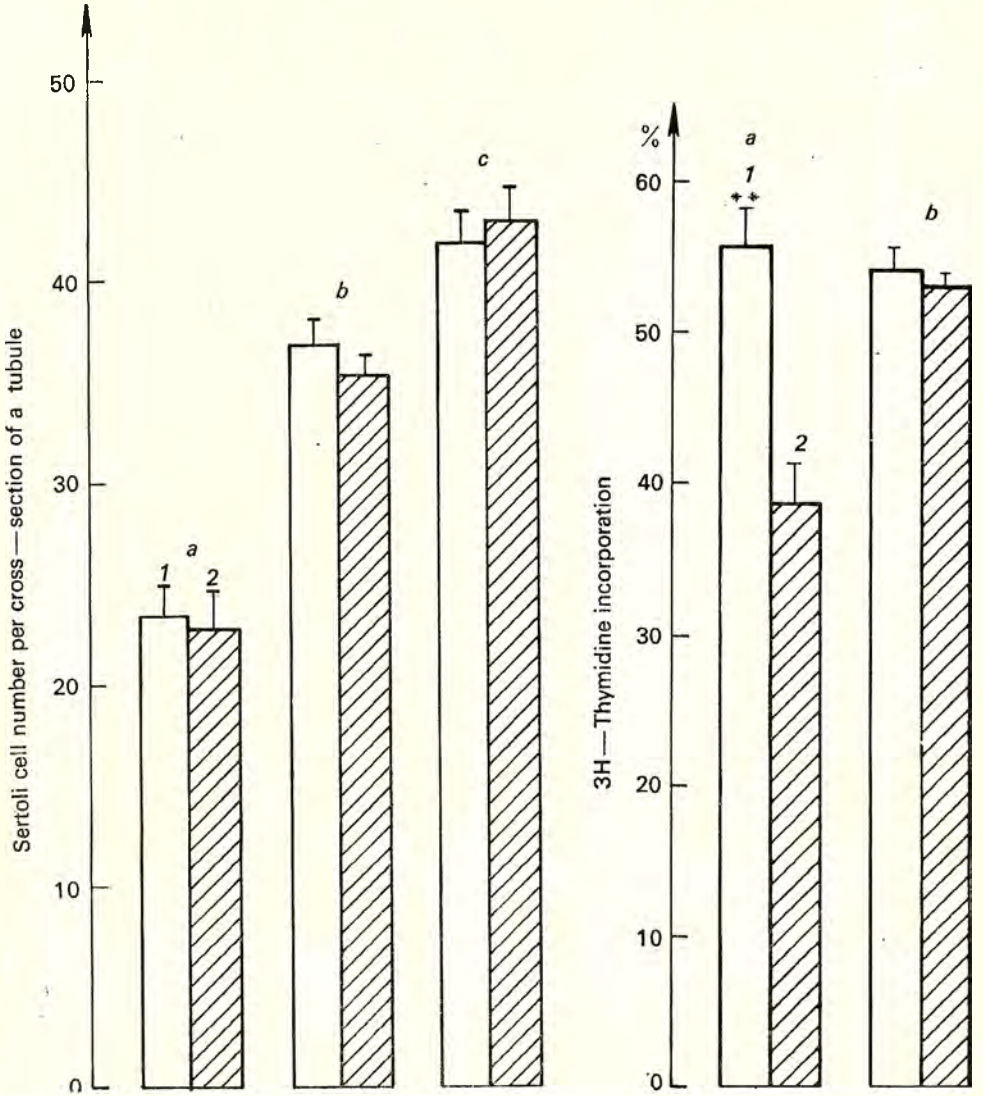

Fig. 2. Sertoli cell number per cross-section of a seminiferous cord and tubule of 6- (*a*), 12- (*b*) and 20- (*c*) day-old control (*1*) and VAD (*2*) rat testes. Data represent the mean±SD. The differences between VAD and control means are not significant ($p<0,1$)

Fig. 3. ^{3}H-Thymidine labelling index of germ (*a*) and Sertoli (*b*) cells of 6-day-old control (*1*) and VAD (*2*) testes. Data represent the mean±SD; ** — $p<0,01$

number showed that in controls spermatocytes predominated as a result of normal germ cell differentiation whereas in VAD specimens spermatogonia prevailed. A considerable reduction of different types germ cells in experimental animals at this age was established. With several time decrement in spermatocytes number, that of In- and B-spermatogonia was rather reduced whereas A-spermatogonia decreased in a lesser extent (Fig. 5).

Ultrastructural observation on 6-day-old VAD testes showed a normal structure of seminiferous cords — Sertoli cells lay on the basement membrane and

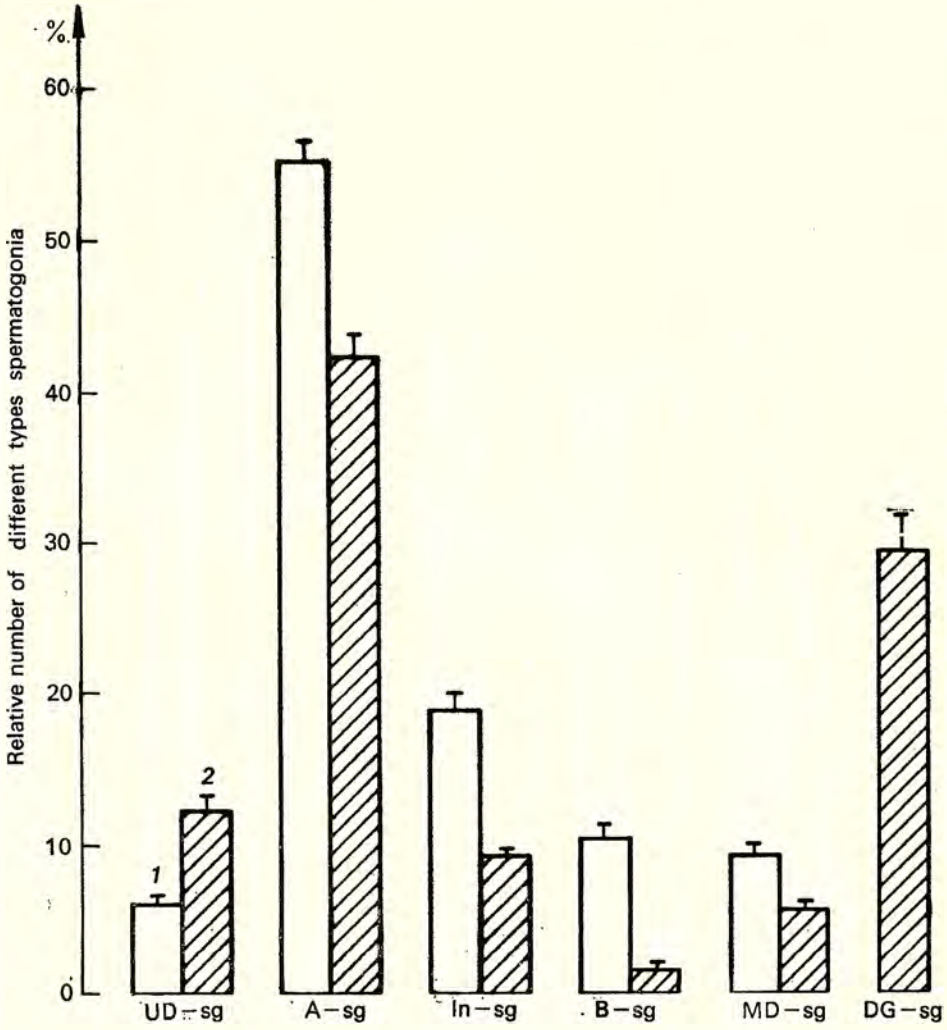

Fig. 4. Relative number of different types spermatogonia of 12-day-old control (*1*) and VAD (*2*) testes

UD-sg — undifferentiated spermatogonia; A-sg, In-sg and B-sg — types A-, In- and B-spermatogonia; MD-sg — mitotic dividing spermatogonia; DG-sg — degenerating spermatogonia. Data represent the mean ± SD; $p<0,01$

prespermatogonia were centrally situated. At this age a normal Sertoli cell structure was visible. Simultaneously with intact prespermatogonia at some places degenerating germ cells connected with wide cytoplasm bridges were observed (Fig. 6). In their cytoplasm many vacuoles were found. In 12- day-old VAD testes the intact Seroli cells and great number of degenerating dividing germ cells was established. They ultrastructurally were visualized as large spherical cells in which cytoplasm numerous vacuoles were seen (Fig. 7). Degenerated germ cells were fagocytozed by Sertoli cell cytoplasm.

In 20-day-old VAD seminiferous tubules spermatocytes were rarely found and some of them showed different degrees of degeneration. Spermatogonia for-

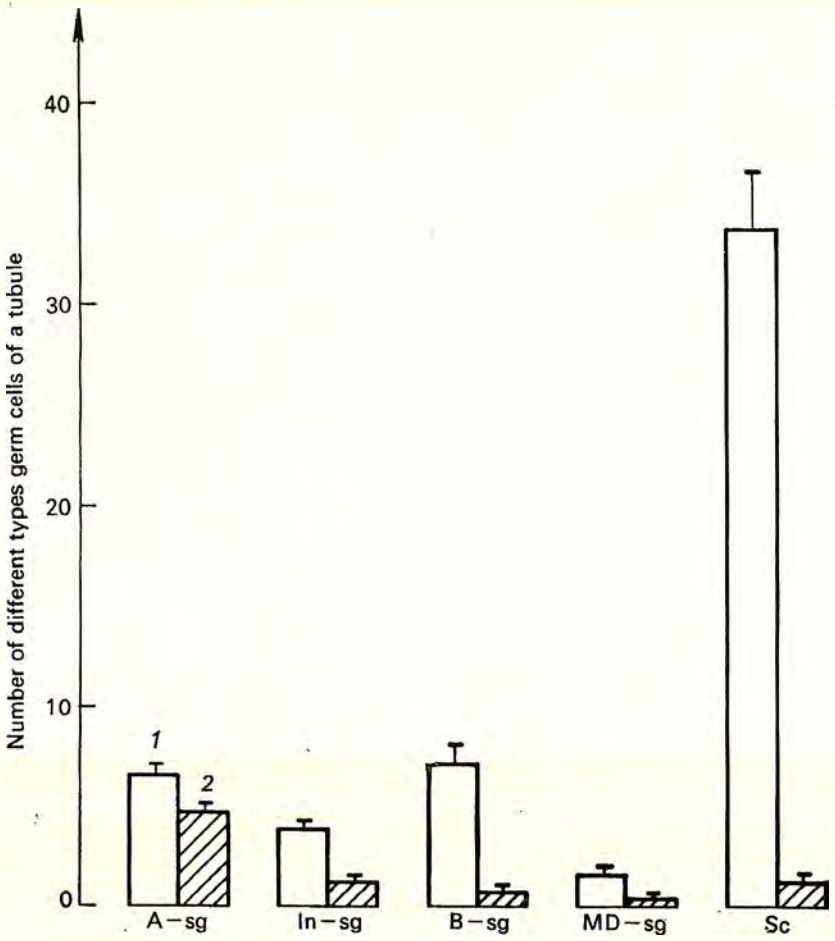

Fig. 5. Number of different types germ cells per cross-section of a seminiferous tubule of 20-day-old control (*1*) and VAD (*2*) testes

A-sg, In-sg and B-sg — types A-, In- and B-spermatogonia; MD-sg — mitotic dividing spermatogonia; Sc — spermatocytes. Data represent the mean ±SD; $p<0,01$

med a heterogeneous population. Certain cells showed varying degenerative features such as irregular nuclear contures, disruption of nuclear envelope, picnotic nuclei with condensed chromatin and cytoplasm vacuolization (Fig. 8). At some places binuclear germ cells with abnormal nuclear shape and contures (probably spermatogonia) were seen. At this age simultaneously with germ cells in process of degeneration a considerable number of intact spermatogonia situated on basal membrane were observed. Most of them were type A-spermatogonia whereas In- and B-spermatogonia were infrequently seen.

On 25th day in control testes clearly formed tight junctions between neighbouring Sertoli cells were visualized while in VAD gonads a typical morphology of these contacts was not seen and desmosome-like structures were found. A disruption of normal seminiferous tubule structure was observed and some spermatogonia which normally lie on the basement membrane were displaced inside to the lumen area. An uncomplete cytotomy of several spermatocytes was demonstrated by wide cytoplasm bridges (Fig. 9).

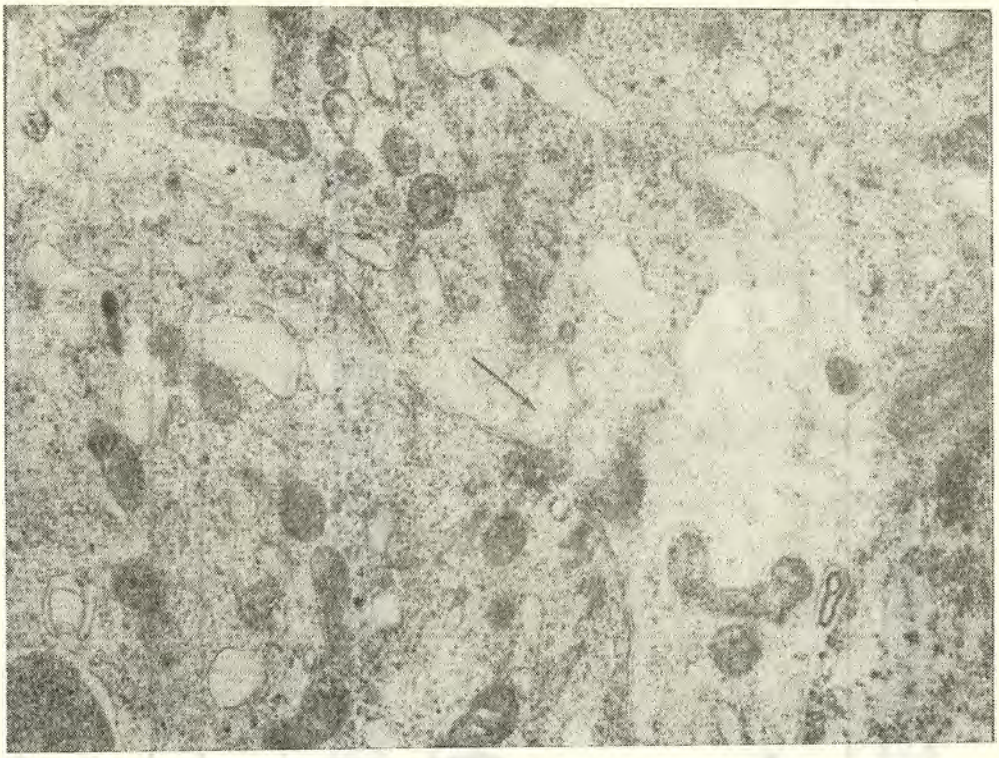

Fig. 6. Electron micrograph (EM) of 6-day-old VAD rat testis. Two degenerative germ cells connected by cytoplasm bridge (arrow), ×28 575

Discussion

During prepubertal rat period on day 4,5 p.p quiescent T_1-prespermatogonia reinitiate their mitotic division and give rise to T_2-prespermatogonia [5]. The latter reduce their size by some consecutive divisions, migrate from seminiferous cord center to the basement membrane and give rise to A-spermatogonia. Our data showed that on day 3th p.p. the number of quiescent T_1-prespermatogonia was unchanged and the decreased prespermatogonial number in 6-day-old VAD testes most probably due to retention of entering mitosis of T_1-prespermatogonia. This suggestion was confirmed by autoradiographic data on day 6th that demonstrated 25 % decrement in ^{3}H-Thymidine germ cell labelling index.

Our investigations on VAD rat testes during prepubertal period (6, 12 and 20 day) showed a considerable reduction of germ cell number in VAD animals comparing with the controls. The extent of this reduction increased with ontogenesis and could be explained with more continuous action of VAD on germ cell proliferation. Our data are in addition to quantitative study of M i t r a n o n d et al. [12] that demonstrated strongly decrease of germ cell number in VAD adult rat testes.

The increased relative number of undifferentiated spermatogonia in 12-day-old VAD testes is probably due to retention of mitotic division of T_1-prespermatogonia and their differentiation into A-spermatogonia. The reduction of absolute and relative number of three types differentiated spermatogonia (according to

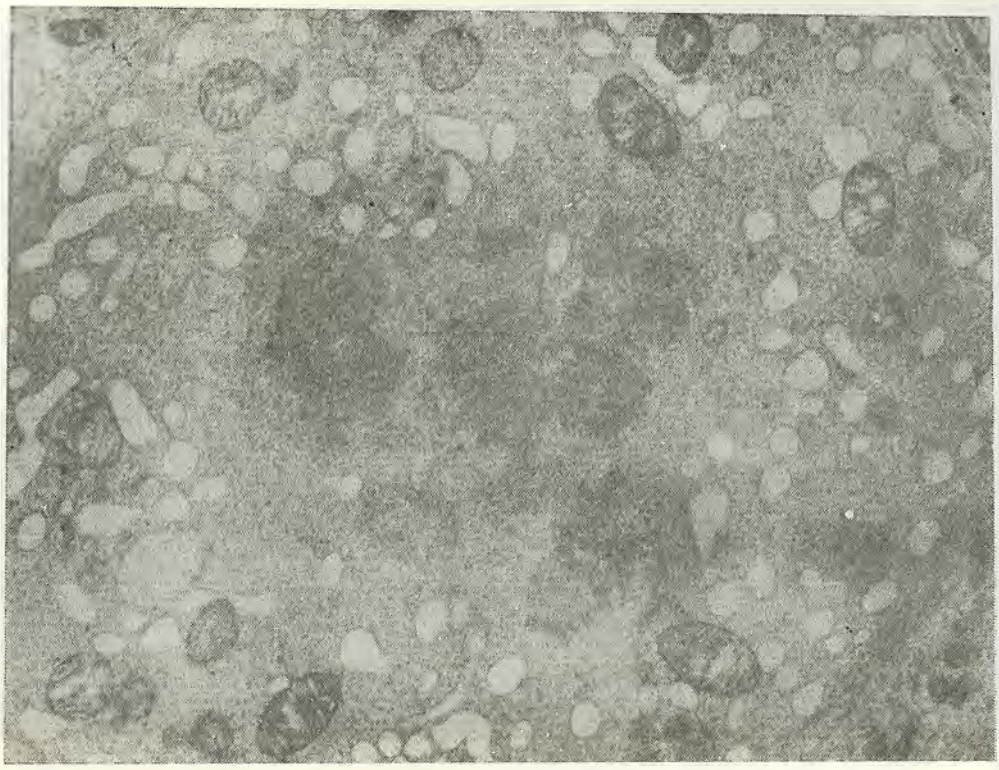

Fig. 7. EM of 12-day-old VAD testis. Cytoplasm vacuolization of a degenerative dividing germ cell, ×16 425

Clermon and Bustos-Obregon [2]) is significant. At this age only in VAD gonads a considerable per cent of degenerating dividing germ cells was established. It is known that in rat by day 12th B-spermatogonia after dividing give rise to preleptotene spermatocytes, which perform the last DNA synthesis and enter meiotic prophase I. That is the second crucial moment (after the first proliferating wave of prespermatogonia on day 5th) during rat prespermatogenesis. Probably at this age the germ cell division at given VAD conditions is rather sensitive stage of germ cell differentiation.

The germ cell counting on day 20th showed that in VAD testes spermatogonia prevailed upon spermatocytes unlike the controls. The considerable reduction of spermatocyte number could be interpreted as a maturation depletion fenomenon that was discussed by Ismail and Moales [10] to explain severe germ cell loss during vitamin A-deficiency. Our quantitation of the three types differentiated spermatogonia demonstrated that in VAD testes on 12th and 20th day A-spermatogonia decreased in a lesser extent comparing with the In- and B-spermatogonia. These data are coincident with results of Ismail et al. [9], Van Pelt and De Rooij (21) that demonstrated a selective mitotic arrest of A-spermatogonia in adult VAD rat testes. This type spermatogonia survives avitaminosis-A and is responsible to reinitiation of spermatogenesis after vitamin A replacement.

The Sertoli cell number per cross-section of seminiferous cord and tubule and their ^{3}H-Thymidine labelling index in prepubertal VAD gonads were unchanged comparing with the controls. It is known that rat Sertoli cells mitotically divide

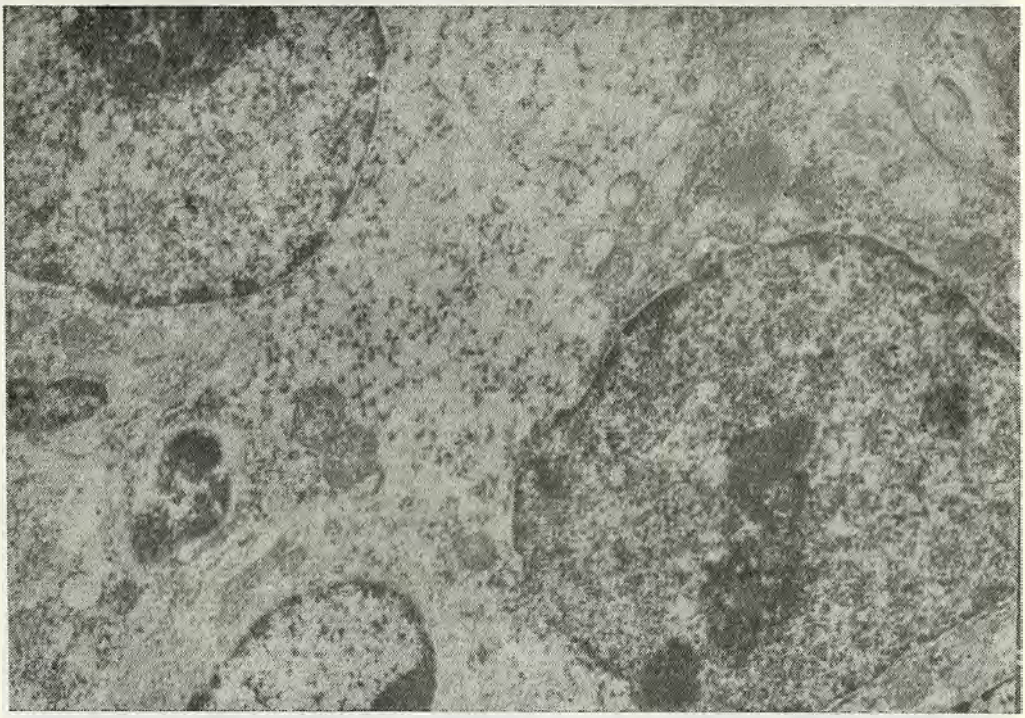

Fig. 8. EM of 20-day-old VAD testis. Cytoplasm vacuolization and onset of degenerative nuclear changes of two B-spermatogonia, × 14 700

by day 15th p.p. after that they stop proliferating and form a constant cell population in the testis [15]. Probably vitamin A-deficiency had no effect on Sertoli cell proliferation during prepubertal period but the question about Sertoli cell functional alterations in that conditions is under discussion.

The ultrastructural observation are in addition to quantitative results and showed destructive changes in germ cells. Similar alterations in germ cell structure were demonstrated by S o b h o n et al. [18] and U n n i et al. [19] in adult VAD rat testes. The absence of typical structure of inter-Sertoli cell tight junctions in 25-day-old VAD testes could be discussed with results of H u a n g et al. [7] demonstrating disruption of these specialized contacts. The recent study of I s m a i l and M o r a l e s [10] showed intact Sertoli cell tight junctions during vitamin A-deficiency. This discrepancy between both investigations is probably due to some differences in VAD status and its effect on Sertoli cell function to form tight junctions.

The decreased germ cell number and ultrastructural changes in VAD testis during prepubertal period could be discussed in the aspects of the functional coupling of immature Sertoli and germ cells and the modulating action of Sertoli cell secreted mitogenic factors. The study of O r t h and B o e h m [16] showed in neonatal rat testes presence of gap junction-mediated communication between Sertoli and germ cells that is of great importance for germ cell development. In earlier study [11] we established that prepubertal rat Sertoli cells secreted mitogenic factors which modulated germ cell proliferation. It is of great interest for us to study whether this Sertoli cell function will be affected by vitamin A-deficiency. According to U n n i et al. [20] VAD caused a great reduction in

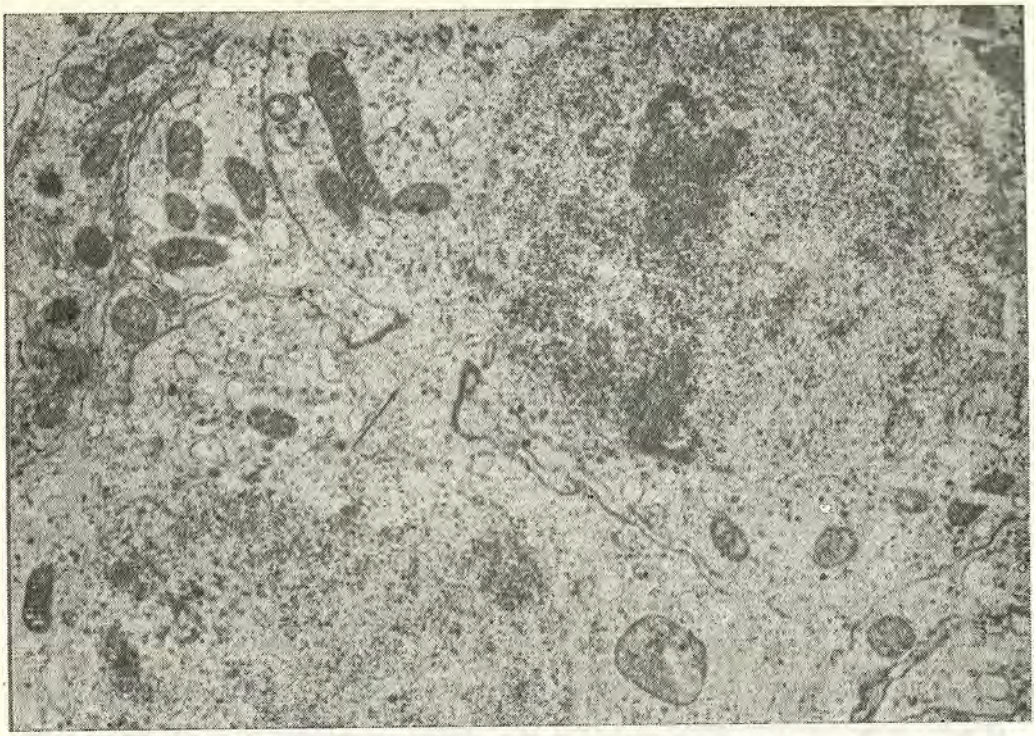

Fig. 9. EM of 25 day-old VAD testis. Two spermatocytes connected by wide cytoplasm bridge (arrow), × 16 425

mitogenic activity of adult testis homogenates. The investigations of Hugly and Griswold [8] and Morales and Griswold [13] demonstrated a significant decrease in the levels of both transferrin and SGP-2 mRNAs in adult VAD rat testes. The contemporary interpretation of our results in terms of Sertoli cell-germ cell interactions requires investigation of Sertoli cell mitogen synthetic function during prepubertal period in conditions of vitamin A-deficiency.

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