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Vitamin A requirements of germ cell types and Sertoli cells in developing rat testis

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Vitamin A deficiency (VAD) was induced in the male offspring of female Wistar rats fed on vitamin A-deficient diet since the first day of pregnancy. A significant reduction of germ cell number, their labelling index and germ/Sertoli cells ratio was established after initiation of gonocyte mitosis. A retardation in tubular lumen and tight junction formation and appearance of spermatid maturation stages were found in VAD testes. Spermatocytes and spermatids undergo more pronounced quantiative and structural changes than spermatogonia. After 12 weeks on a VAD diet a severe regression of seminiferous epithelium was observed and the first spermatogenic wave does not come to completion. The obtained results showed that VAD in developing rat testes severely affects germ cell proliferation and differentiation but not Sertoli cell structure and kinetics that demonstrated the different vit. A requirements of germ cell types and Sertoli cells.

Key words: vitamin A-deficiency, germ cell types, Sertoli cells, developing rat testis.

Introduction

It is known that VAD in adult rat caused a cessation of spermatogenesis, degeneration and loss of germ cells which leads to regression of the seminiferous epithelium [8]. Quantitative studies showed a decrease in number of different germ cell types [12] and a reduction of mitotic activity of spermatogonial cells [18]. According to the studies by [22] there appears to be a selective mitotic division arrest of A_1 spermatogonia which are responsible for reinitiation of spermatogenesis after vit. A replacement [20]. The somatic cell component of the seminiferous epithelium (Sertoli cells) in adult rats seems to be morphologically and quantitatively resistant to VAD conditions [13, 7]. The information about the structure of tight junctions which are the basis of the blood-testis barrier is unclear due to contradictory data from VAD studies [6, 13].

The available information about the effect of VAD on male gametogenesis during the early postnatal period up to sexual maturity, when crucial events occur in

proceeding of the first spermatogenic wave, is rather scarce. Eskild et al. [4] reported that the immature testis has a hight requirements for retinol.

The aim of the present work was to study the alterations in germ and Sertoli cell structure, their kinetics and the relationships between cell types in seminiferous epithelium in conditions of vit. A-deficiency during prespermatogenesis and first spermatogenic wave. In this way the investigation will provide new data on the different vit. A requirements of germ cell types and Sertoli cells during critical periods after birth to sexual maturity.

Material and Methods

The effect of VAD on the developing rat testis was investigated at various ages after birth in the male offspring of female Wistar rats fed on AIN-76 diet without vit. A since the first day of pregnancy. For regular histology the testes were fixed in Serra's fixative and embedded in paraffin. Pieces from each testis were fixed in 2.5% glutaraldehyde, postfixed in 1% OsO_4 and embedded in Durcupan for routine electron microscopy (EM). EM observations and microphotos were made on an Opton EM 109 microscope. Semithin 1 µm sections were stained with Methylene- blue- Azur II-basic Fuchsin and were used for quantitation of germ cell types and Sertoli cells. The overall number of germ and Sertoli cells and the number of different germ cell types were determined on at least 200 cord or tubule cross-sections per animal. The results were expressed as cells per seminiferous cord/ tubule or as percentage from the total germ cell number. Three hours before 4.5-, 6- and 14 day-old VAD and control animals were killed, they received an i.p. injection of 37 kBq/g body weight of ^{3}H-thymidine (spec. act. 1.1 TBq $mmol^{-1}$). The paraffin sections were processed for autoradiography using liquid nuclear emulsion Ilford K_2. The percentage of labelled germ and Sertoli cells was determined in 100 cord cross-sections from each animal. For statistical analysis Student's t-test was used.

Results

Histological and EM observations on testes from 3- and 6-day-old VAD testes showed a normal structure of the seminiferous cords. The appearance of the most advanced differentiated spermatogonia (type A-, In- and B-spermatogonia, according to Clermont and Bustos-Obregon [3]) in VAD animals did not differ from the controls. The first differentiated A spermatogonia along the basement membrane were identified on day 7th, In spermatogonia - on day 9th and B spermatogonia - on day 10th after birth. Preleptotene spermatocytes appeared on 12th day, leptotene spermatocytes - on 13th day in VAD and control rats. In seminiferous cords of 14-day-old controls few early pachytene spermatocytes could be found whereas in VAD testes at this age the most advanced type of meiotic germ cells had reached the zygotene stage. Only in 12-day-old VAD testes a great number (about 20%) of degenerating mitotic germ cells was established. They had a tendency of a more central position in the cords but were not found on the basement membrane (Fig. 1*b*). Ultrastructurally the degenerating mitotic germ cells appeared as large spherical cells with swollen endoplasmic reticulum and enlarged mitochondria (Fig. 1*a*) In 20-day-old control rats tubular lumen was already present and numerous primary spermatocytes were observed in the seminiferous tubules (Fig. 2*a*). In VAD animals of the same age the lumen was not formed yet and only few primary spermatocytes were found in a small number of

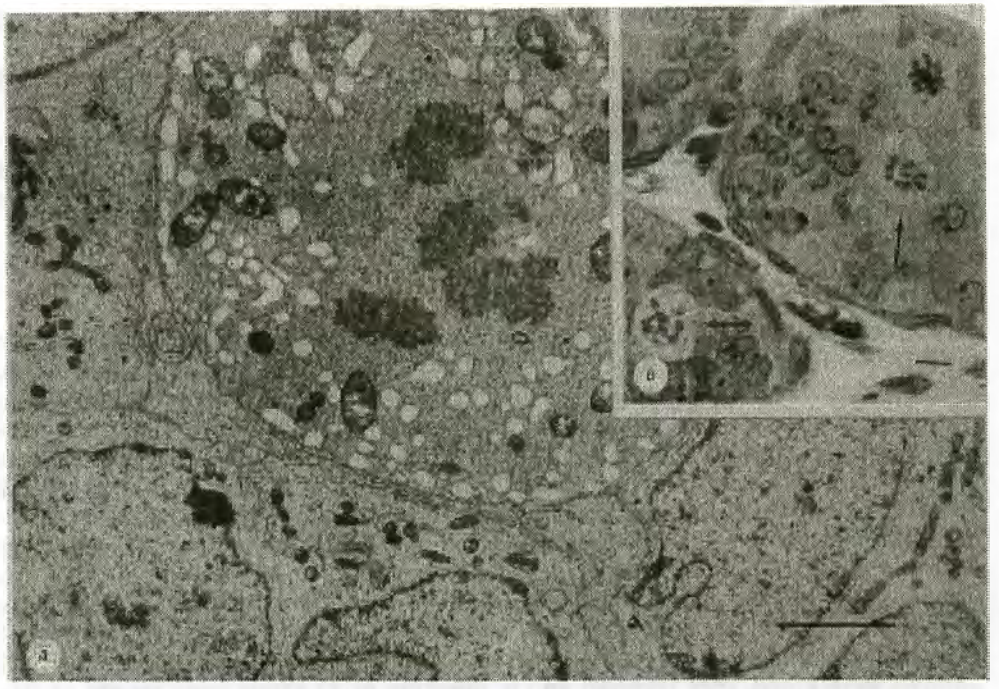

Fig. 1. Photomicrographs of 12-day-old VAD rat testis
a — electron micrograph of degenerating mitotic germ cell (×3300);
b — semithin section of seminiferous cords. The degenerating mitotic germ cells are situated in the centre of the cords (arrows) (× 800). Bar 2.4 μm

seminiferous cords (Fig. 2*b*). On day 25th some round spermatids could be seen in the control testes. In VAD gonads the tubular lumen was formed at day 30th and the first round spermatids were found on day 34th. In 40th day-old controls elongated spermatids were present whereas in seminiferous tubules of VAD animals the most advanced germ cells were round spermatids. In control rats the spermatogenesis was completed by day 65th as all stages of the cycle of the seminiferous epithelium had a full complement of germ cell generations (Fig. 3*a*). In VAD animals the first spermatogenic wave does not come to completion i.e. no spermatozoa were found. On day 65th the testes showed a thin seminiferous epithelium due to degeneration and loss of germ cells. Most of the seminiferous tubules contained only Sertoli cells, a few spermatogonia and some primary spermatocytes (Fig. 3*b*). The regression of seminiferous epithelium was accompanied with a significant decrease of body weight (up to 55% of control value), testis weight (up to 70%) and tubular diameter (up to 40%).

EM study on VAD animals revealed that spermatids and spermatocytes underwent more pronounced structural alterations than spermatogonia. Binuclear germ cells - spermatogonia, spermatocytes and spermatids were also seen in VAD animals. By day 25th Sertoli cells were morphologically mature in both of the investigated groups. Between neighbouring Sertoli cells a typical structure of tight junctions was seen only in controls whereas in 25-day-old VAD testes desmosome-like contacts were found. These specialized inter-Sertoli cell structures were observed at 34th day in VAD animals.

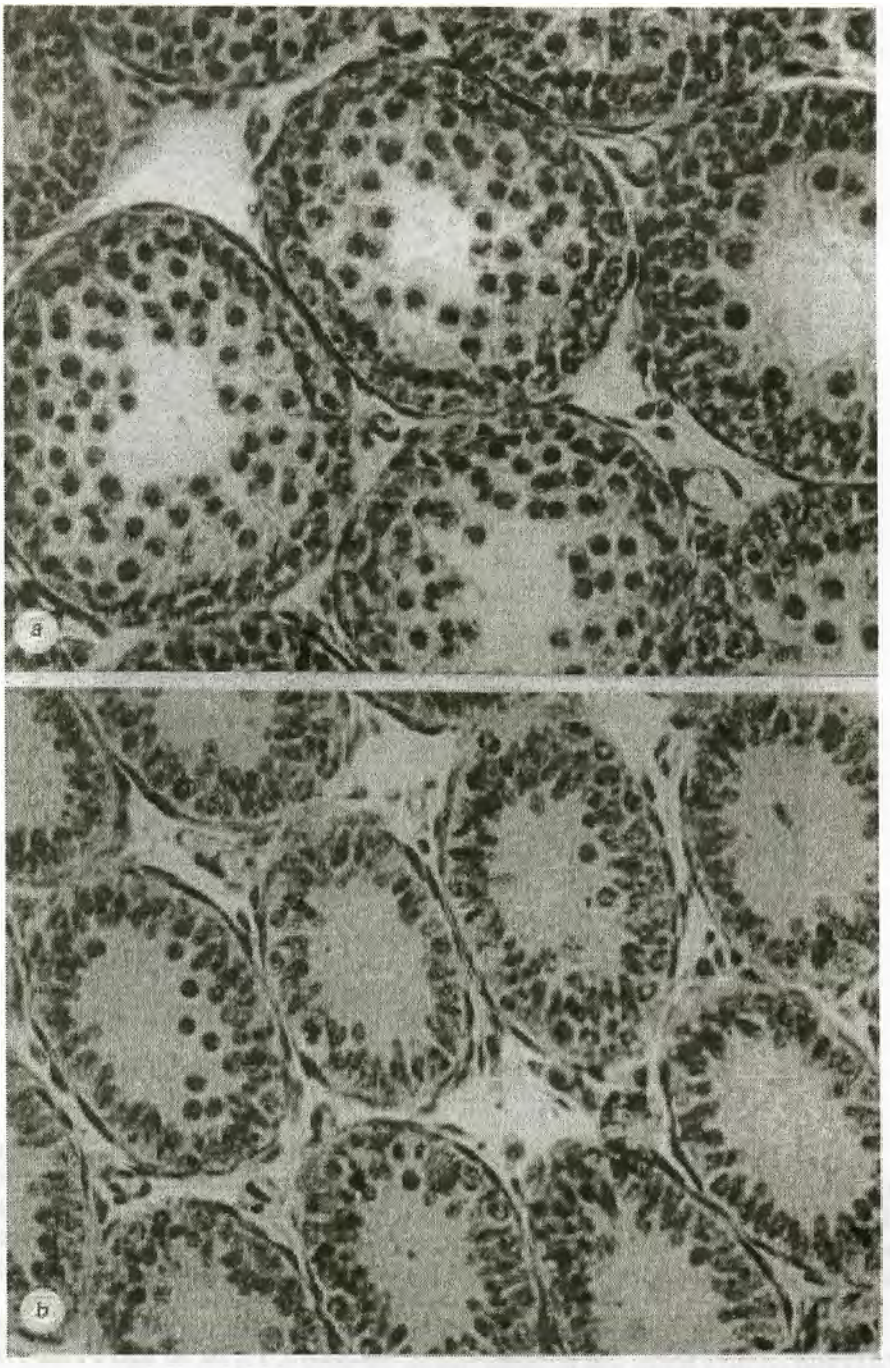

Fig. 2. Photomicrographs of the cross-section of rat seminiferous tubules of 20-day-old control (a) and VAD (b) testis (× 500)

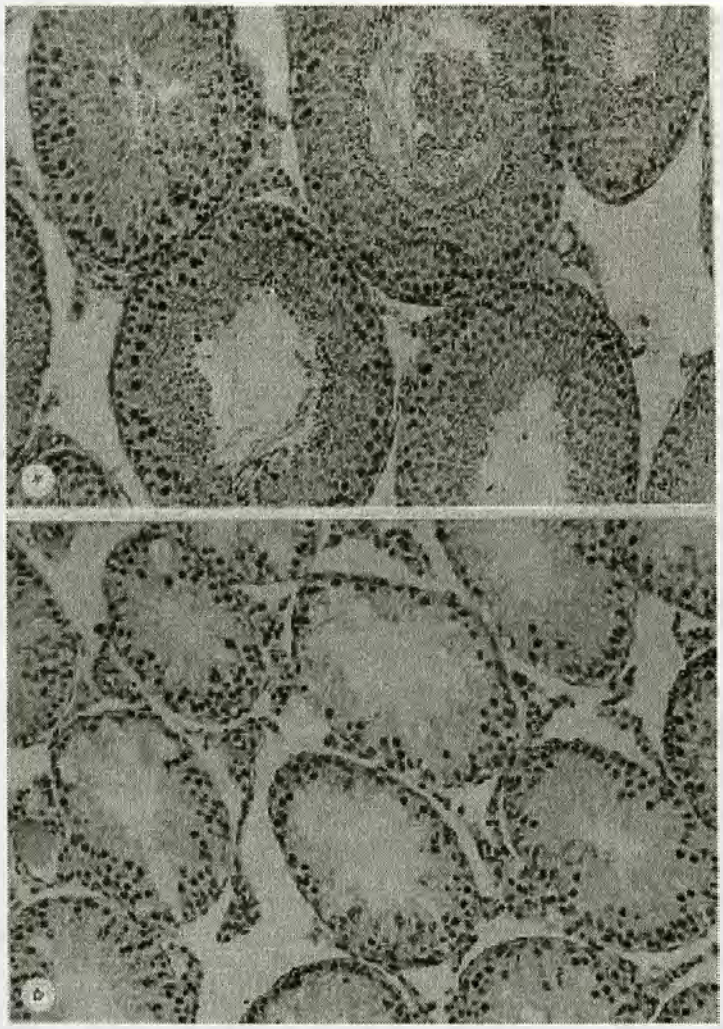

Fig. 3. Photomicrographs of the cross-section of rat seminiferous tubules of 65-day-old control (a) and VAD (b) testis (× 125)

The labelling index of germ cells under vit. A-deficient conditions was significantly lower comparing to the controls (Fig. 4). The incorporation of ^{3}H-thymidine on day 4.5 was 3-fold decreased. Germ cell labelling indices on day 6 and 14 were respectively 30% and 40% lower than the control values ($p < 0.01$). The difference between the labelling index of Sertoli cell from VAD and control animals on day 6 was not significant.

On day 3 p.p. the difference of the number of germ cells per cord cross-section in VAD and control testes was nonsignificant. On days 6, 12, 20, 25 and 34 after birth there was a reduction of the overall number of germ cells by about 2-, 3-, 4-, 4.5- and 5-fold respectively as compared to the controls (Fig. 5). The Sertoli cell number per

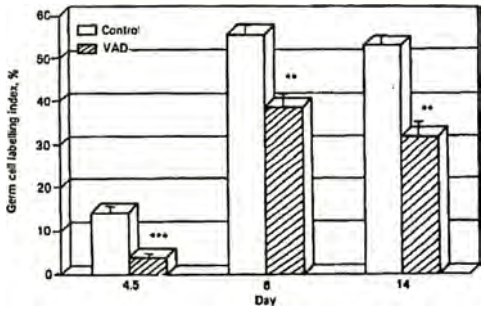

Fig. 4. ^{3}H-thymidine labelling index of germ cells of prepubertal control and VAD testes. Data represent tje mean ± SD; n=3). Asterisks, denote values significantly different from control rats (** $p < 0.01$; *** $p < 0.001$)

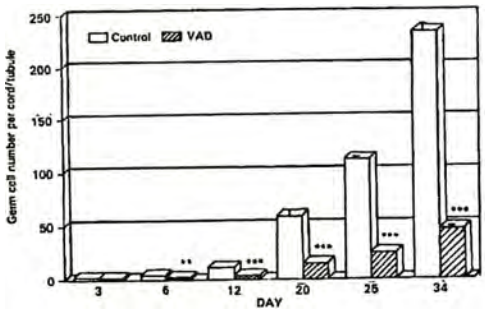

Fig. 5. Overall germ cell number per cross-section of a seminiferous cord/tubtle in control and VAD developing rats from day 3 to day 34. Data represent the mean ± SD; n=3). Asterisks denote values significantly different from control rats (** $p < 0.01$; *** $p < 0.001$).

cord/tubule cross-section during investigated time points showed nonsignificant differences between both groups. The germ/Sertoli cell ratio showed a significant rise in the controls, whereas in VAD animals the slight increase of this ratio was found (Fig. 6).

The quantitation of different germ cell types revealed that on day 7 undifferentiated primitive type A spermatogonia [24, 1] predomonated in VAD testes, whereas differentiated A spermatogonia prevailed in the controls. On days 8, 9, 10 and 12 the percentage of primitive type A spermatogonia in VAD animals was about 2-3-fold higher but the percentage of differentiated A-, In- and B-spermatogonia was lower than the controls. A significant increase in ratio of differentiated/undifferentiated spermatogonia in the controls was not observed in VAD animals where a slight increment of this ratio was found (Fig. 7). On day 12th the percentage of the sum of In- and B-spermatogonia was two times lower whereas the percentage of differentiated A spermatogonia was decreased only by approximately 20%. In 14-day-old VAD testes the percentage of primary spermatocytes was 5-fold lower than the control value. The ratio of spermatocyte to spermatogonial number showed that during puberty spermatocytes predominated already on day 20, reaching (53%) from the overall germ cell population whereas in 20- and 25-day-old VAD rats it was spermatogonia that still prevailed (66% and 57% respectively). On day 34 the percentage of the sum of spermatocytes and spermatids in VAD testes was higher than that of spermatogonia (Fig. 8).

Quantitation of different germ cell types per tubular cross-section on days 20, 25 and 34 demonstrated a more pronounced decrease (6-fold) of the number of more advanced germ cell types (spermatocytes and spermatids) than that found for spermatogonial cells. The number of B- and In-spermatogonia was reduced significantly (4- and 3-fold respectively), whereas the number of A-spermatogonia decreased to a lesser exent (1.5 - 2-fold) (Fig. 9).

Discussion

In 4.5-day-old neonatal rats quiescent prospermatogonia resume mitotic division after a resting period of about 10 days and give rise to primitive type A-spermatogonia. The newly formed germ cells also start mitotic division, accompanied by a re-

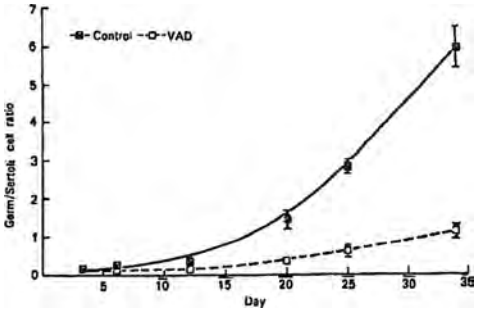

Fig. 6. The germ/Sertoli cell ratio in developing control and VAD testes from day 3 to day 34. Data represent the mean ± SD. The differences between VAD and control values are significant for each time point except these for day 3 ($p < 0.01$)

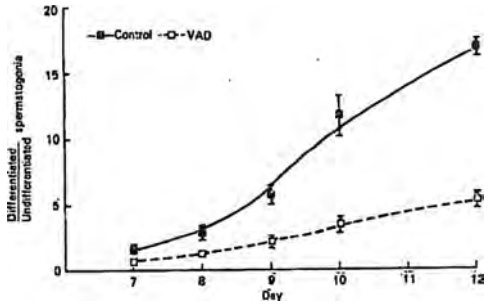

Fig. 7. The ratio of differentiated/undifferentiated spermatogonia in prepubertal control and VAD testes from day 7 to day 12. Data represent the mean ± SD. The difference between VAD and control value are significant for each time point ($p < 0.01$)

duction in size, while migrating from the centre to the periphery of the seminiferous cords give rise to differentiated A-spermatogonia [24]. We found that the number of quiescent prospermatogonia in 3- day-old VAD rat testes is practically equal to that in controls, whereas the germ cell labelling indices on day 4.5 and 6 p.p. were significantly lower than those established in controls. The 2-fold decrease in germ cell number per cord cross-section on day 6 is most probably due to retention of entering mitosis of prospermatogonia and their differentiation to A-spermatogonia. Busulphane treatment carried out during the resting period of prospermatogonia delayed the expected resumption of mitosis on the 4.5 day by more than 10 days [23]. We could suggest that the changes of germ cell kinetics in early postnatal period is due to an effect of early vit.A deficiency on the proliferation of germ cell and on the initiation of their first postnatal mitotic wave which is a crucial event in establishment of spermatogenesis after birth. Another critical event in the first spermatogenic wave in rat is the initiation of meiosis on day 12 p.p. The presence of degenerating germ cell mitoses found in the centre of the seminiferous cords at the same time point can be taken as an indication that under VAD conditions some of the germ cells did not succeed to move and relocate on the basement membrane. Since germ cells which remain central in the cords have been reported to degenerate it has been suggested that the change of position of germ cells and their contact with the basal lamina are vital for their survival and for establishment of spermatogenesis [11].

The decrease of spermatogonial labelling index of VAD testes from 14-day-old rats idicates that after early postnatal events VAD probably continues to affect germ cell proliferation up to end of prepubertal period. The reduced germ cell labelling indices we found in prepubertal VAD rats is consistent with the results obtained by Unni et al. [18] who reported a significant decrease of the spermatogonial mitotic index. Quantitation of different germ cell types in prepubertal VAD animals revealed a marked decrease in ratio of differentiated/undifferentiated spermatogonia leading to defferent patterns of its control and VAD curves. The percentage of primary spermatocytes, B- and In -spermatogonia significantly declined, whereas the decrease of the number of A spermatogonia was much less pronounced. Therefore we can suggest that vit. A deficiency can bring about retardation both of the premeiotic spermatogonial differentiation and entering meiosis.

Under VAD conditions there is a gradual reduction of the overall germ cell number per cord/tubule cross-section and subsequent decrease in germ/Sertoli cell ratio in the course of the first spermatogenic wave. Our histological studies on pubertal VAD testes reveal a retardation of the appearance of spermatid maturation stages. The percentage of the sum of spermatocytes and spermatids in VAD rats began to prevail upon that of spermatogonia about 10 days later than the controls. The arrest of spermatogenesis at preleptotene stage in adult VAD rats suggests that normal progression of meiosis beyond preleptotene spermatocytes cannot be achieved without vitamin A [5]. Our findings suggest that during puberty VAD affects meiotic and postmeiotic germ cell development.

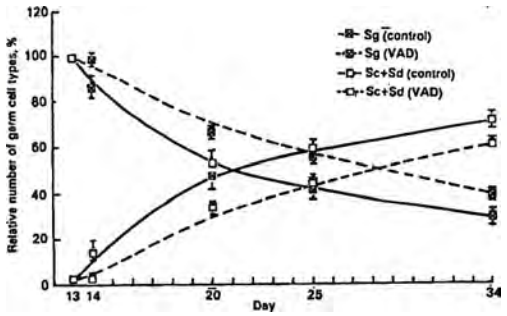

Fig. 8. Pescentage of different germ cell types in pubertal control and VAD rats from day 13 to day 34. The differences between VAD and control value are significant for each time point except these for day 13 ($p < 0.01$). Sg - spermatogonia; Sc - spermatocytes; Sd- spermatids

Our results on the number of different germ cell types per tubular cross-section and EM observation on pubertal VAD testes demonstrated that spermatocytes and spermatids underwent more considerable changes in their number and structure than spermatogonia. These results suggest that germ cells at later stages of spermatogenesis are affected by vit. A deficiency to a greater extent than those at earlier stages which are consistent with the assumption of Mitanond et al. [12] for adult rat testis. It was reported that some chemical agents, heat, and certain pathological conditions

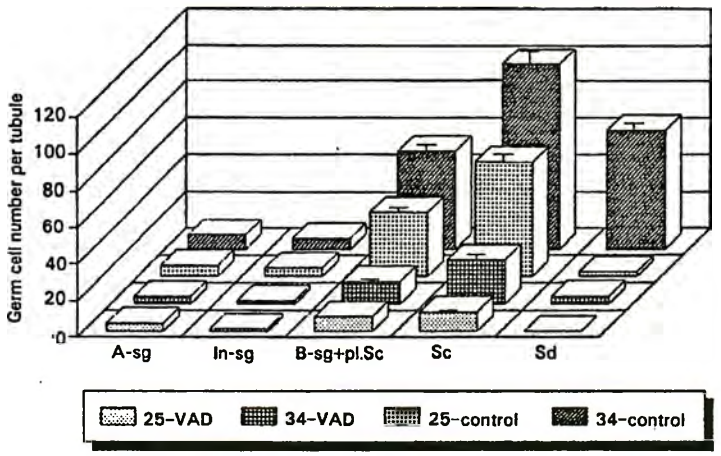

Fig. 9. Number of different types germ cells per tubular cross-section in 25- and 34-day-old control and VAD rats. Data represent the mean ± SD. A-sg, In-sg and B-sg - A-, In- and B-spermatogonia; pl.Sc - preleptotene spermatocytes; Sc - spermatocytes; Sd - spermatids

also selectively affect more differentiated germ cell stages [9]. The considerable decrease of the number of spermatocyte and spermatids established by us, which is apparently due to the decreased number of In- and B-spermatogonia, can be interpreted as a results of the maturation depletion phenomenon leading to severe atrophy of seminiferous epithelium. Furthermore in VAD animals the first spermatogenic wave does not come to completion, i.e. there is no formation of spermatozoa. Recently Ismail and Morales [7] explained the mode of seminiferous epithelium regression in adult VAD testes with degeneration of germ cell at all steps of spermatogenesis and with a germ cell maturation depletion process.

Our finding that the number of A-spermatogonia decreased to a lesser exent compared to the more advanced germ cell types characterizes this type of spermatogonia as a cell population that is more resistant to vit. A deficiency. This type of spermatogonia survives avitaminosis A and is responsible for the reinitiation of spermatogenesis after vit. A replacement [8, 19].

The Sertoli cell number per cord/tubule cross-section and their ^{3}H-thymidine labelling index in prepubertal and pubertal gonads remain unaffected under VAD conditions. It is known that rat Sertoli cells proliferate up to 15 day p.p. [14]. Thereafter mitotic division ceases and they acquire the morphological characteristics typical for mature Sertoli cells [17]. Our results showed that proliferation and maturation of the Sertoli cells occur normally in VAD conditions, which characterized them as a relatively stable component of the seminiferous epithelium. It has been reported that after severe germ cell depletion in adult VAD testes Sertoli cells remain in seminiferous tubules and preserve their number [13] and structure [7]. We found that in VAD animals inter-Sertoli cell tight junctions morphologically appear later than in control animals. In VAD rats this event coincides with a delayed formation of the tubular lumen. Pharmacological studies have shown that gonadotropin depletion with oestradiol benzoate or chlomiphen delays the establishment of the blood-testis barrier by about 10 days [21, 2]. The coincidence of the delay of lumen formation and tight junction appearance found by us, can be interpreted in the light of the data of Pelletier and Bayer [15] who demonstrated that attainment of barrier competence coincides with the completion of tubular lumen development.

The present study reveals that in immature rat VAD severely affects the structure of germ cell their proliferation and differentiation which lead to a disturbance of the first spermatogenic wave. In a previous study [10] we found that VAD inhibits the mitogenic effect of proteins secreted from prepubertal rat Seroli cells. Despite unchanged Sertoli cell kinetics in developing VAD rat testis the above mentioned data about germ cell alterations correspond with the reduced mitogenic activity of prepubertal Sertoli cells from VAD testes. The obtained results suggest that germ cells are more sensitive to vitamin A deficiency than the Sertoli cells and the individual germ cell types also have different requirements for vit. A. These data are consistent with the assumption of Pescovitz et al. [16] that vit. A plays a crucial role for germ cell proliferation and differentiation and normal proceeding of spermatogenesis.

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