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Long-Term Impact of Acute Retinoic Acid Supplementation at the Young Age on Testicular Architecture of Wistar Albino Rats

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Abstract

Introduction: Inappropriate and excess vitamin supplementation, particularly for vitamin A, is increasingly recognized as a public health problem in developed countries. On the other hand, blind supplementation of vitamin A, for children in developing countries is a subject of controversy in the literature. The crucial role of vitamin A in the process of spermatogenesis in adult rodents is well established, but only a few publications are consecrated to the long-term effect of vitamin A intake at a young age on testicular development and differentiation. **Objectives:** Our study aimed to evaluate the long-term effects of acute supplementation at an early age, in the post-natal period, on spermatogenesis and testicular trophicity at adult age. **Material and Methods:** Young Wistar Albinos rats of 22 days received an acute high dose of supplementation of vitamin A (retinyl palmitate). The control group, group 1, received only extra virgin olive oil, Group 2 a dose of 7000 IU/kg of retinyl palmitate, group 3, 14,000 IU/kg, and Group 4 a dose of 28,000 IU/kg. At 10 weeks of age, the testes' testosterone levels were measured by ELISA. For histological assessment, sections were stained with Hematoxylin eosin, and the Johnsen score was used to evaluate spermatogenesis in the seminiferous tubules. **Results:** The average testicular weights of rats were significantly lower in group 4 ($p < 0.05$), and so was the testosterone level in the testis compared to the control group ($p < 0.01$). Most of the seminiferous tubules were concerned by an arrest of spermatogenesis and the Johnsen score was decreased with a mean score of 5.96 ± 1.60 ($p < 0.001$) in that Group. In Group 3, Johnsen's score was significantly better than the one obtained with the control. **Conclusion:** We observed a negative effect in the long term with a high acute

dose of supplementation of retinyl palmitate at a young age, on testicular development and differentiation. Despite a return to normal diet after that supplementation, during childhood, impaired spermatogenesis was identified at the adult age with an arrest of spermatogenesis. The reversibility of that lack of differentiation by a return to a normal diet is questionable and would need more investigation.

Keywords

Vitamin A, Retinyl Palmitate, Spermatogenesis, Testis-Wistar Albino Rats

1. Introduction

The cumulative effect of a diet enriched with vitamin A and the use of self-prescribed vitamins is responsible for negative metabolic effects that are not always diagnosed in time. Vitamin A is fat-soluble and can be stored in the body, particularly in the liver. This phenomenon is increasingly recognized as a public health problem [1]. However, little is known about the precise mechanism of toxicity on male reproduction in the event of excess vitamin A [2]. Some authors demonstrate that in well-nourished individuals, prolonged intake of vitamin A can increase mortality [1].

It is well known that vitamin A plays a crucial role in spermatogenesis. Its active metabolite, retinoic acid, is essential for the differentiation of spermatogonia and the initiation of meiosis [3] [4]. Spermatogenesis is blocked at the stage of spermatogonia in rats with vitamin A deficiency and, more precisely, in the transition from undifferentiated spermatogonia to the stage of differentiated spermatogonia before entering mitosis and then in meiosis [5].

Vitamin A deficiency remains a public health challenge in certain regions of the world, facing malnutrition and famine. According to the WHO, this deficit affects approximately 190 million 5-year-old children in Africa and Southeast Asia [6]. To deal with this problem, many supplementation programs have been initiated, and the WHO has also deployed a vitamin A supplementation regimen between 6 and 11 months with a single dose ([7] [8]) of 100,000 IU to 200 000 UI every 6 months between 6 months and 59 months.

With the identification of the harmful effects of excessive vitamin A intake, this regimen has become a subject of controversy, and there is currently a debate about the relevance of vitamin A supplementation in young children [9].

Only a few publications are dedicated to the long-term effect of excess vitamin A on spermatogenesis [10], and the authors, Yokota *et al.* [10], highlighted the negative effects of chronic supplementation on spermatogenesis. Considering the debate over vitamin A supplementation in children in developing countries, our work focused on the long-term effects of acute supplementation at an early age on spermatogenesis and testicular trophicity in adulthood. To assess the results, we used an animal model, the Wistar albinos rat.

2. Material and Methods

2.1. Animals

Four healthy pregnant female albino rats of the Wistar type, from the same strain and generation, were obtained from the animal facility of the Toxicology and Hydrology Laboratory of the Faculty of Medicine, Pharmacy, and Dentistry of the Cheikh Anta Diop University of Dakar. On the day of birth (day 0), twelve pups were selected according to their weight to avoid fluctuations in the pups' dietary intake.

Food and water intake were normalized to avoid significant variation in rat growth. Throughout the experiment, the animals were fed a normal commercial pellet diet and given water ad libitum. After 21 days, 12 male rats were selected and randomly divided into four groups.

Vitamin A was given on day 22. We used the scheme of retinyl palmitate supplementation for infants recommended by the WHO, concerning developing countries, with a recommended dose of 100,000 UI and 200,000 UI between the ages of 6 and 59 months. Capsules of vitamin A were obtained from the Unity of Pediatrics at a health center 30 km from the university in the sanitary district of Dakar. We diluted the liquid from the capsule with virgin oil to obtain the equivalent dose of 100,000 UI for 7 kg, the mean weight of a 6-month-old baby. (*i.e.*, 14,286 UI/kg). The distribution was performed as follows and was administered to the rats by gavage.

Group 1: administration of extra virgin olive oil.

Group 2: administration of a dose of 7000 IU/kg of retinyl palmitate.

Group 3: administration of a dose of 14,000 IU/kg corresponding approximatively to the dosage of the capsule of 100,000 UI founded in the program supplementation in pediatrics.

Group 4: administration of a dose of 28,000 IU, corresponding approximately to the dose of 200,000 UI also administered in the supplementation program.

The different doses all rats were fed for 8 weeks in cages maintained at 23˚C ± 2˚C with a light/dark cycle of 12 hours. The rats' body weights were measured weekly. Food consumption and the volume of water consumed were measured daily.

After administration of vitamin A to the rats, special attention was paid for the first 4 hours to record any physical changes and signs of intoxication. At 10 weeks of age, the rats were fasted overnight and then sacrificed under sodium pentobarbital euthanasia. The reproductive organs of male mice were removed just after.

The study was carried out following Guideline 408 of the Organization for Economic Co-operation and Development (OECD), revised and adopted on June 25, 2018, on the testing of chemicals and according to the regulations of the Charter on the Protection of Animals [11]. The study also received agreement from the ethical committee of the institution.

2.2. Methods

2.2.1. Testosterone Levels in the Testis

Testicular tissues previously frozen at −80˚C were cut into thin sections, crushed on a bed of ice, and homogenized in a mixture of 5% Triton X-100 and phosphate with a 1:1 ratio and at a concentration of 10 µL/mg of tissue until the tissue pieces are entirely dissolved. The homogenized tissue was centrifuged at 9000 rpm for 15 minutes. The supernatant was used to measure the testosterone level in the testes using an Elisa test (enzyme-linked immunosorbent assay) according to the manufacturer's protocol provided by the Testosterone Elisa kit (Testosterone ELISA, Human®).

2.2.2. Histopathological Examination of the Testicles

The testes were fixed with modified Davidson's fluid fixative (10% formalin of 37 - 40% formaldehyde, 15% ethanol, 5% glacial acid, and 50% distilled water) [12]. Samples were treated with graduated alcohol of increasing degree, then in xylene and included in paraffin. Sections of tissue were made, then dewaxed and stained with hematoxylin and eosin (EO). The slides were mounted using a synthetic resin (glue Eukitt®) and air-dried before microscopic examination.

To obtain the Johnsen score, slides were examined under an optical microscope (magnification, ×100) using light microscopy (Leica DM2500). Twenty randomly selected sections of each testis were used for morphological examination. In each slice, 10 seminiferous tubules were assigned a score, resulting in a total of 200 seminiferous tubules. The result was summed across the different scores and then divided by the number of evaluated tubules, giving the final Johnsen score [13]. Johnsen's criteria are based on a ten-point scoring system for quantifying spermatogenesis according to the profile of the cells encountered along the seminiferous tubules (Table 1). Johnsen's score of 10 indicates maximum

Table 1. Johnsen score.

Score	Explanation
10	Full spermatogenesis
9	Many late spermatids, disorganized tubular epithelium
8	Few late spermatids
7	No late spermatids, few early spermatids
6	No late spermatids, few early spermatids, arrest of spermatogenesis at spermatid stage, disturbance of spermatid differentiation
5	No spermatids, many spermatocytes
4	No spermatids, few spermatocytes, arrest of spermatogenesis at the primary spermatocyte stage
3	Spermatogonia only
2	No germ cells, Sertoli cells only
1	No seminiferous epithelial cells, tubular sclerosis

spermatogenesis activity, whereas a score of 1 indicates a complete absence of germ cells. The method is practical and easy to perform, providing a connection between the results of seminal analyses and those of testicular biopsies [14].

3. Statistical Analysis

The parameters of the different groups were presented as an average standard deviation of the number of animals used for each group. The results were analyzed using the Wilcoxon-Mann-Whitney test. A p-value ≤ 0.05 was considered statistically significant. The data was processed and analyzed using SPSS software.

4. Results

4.1. Body Mass

On day 22, when vitamin A was administered, the average weight of the rats was 17.55 ± 4.33 g. From the 5th week (day 35), significant growth was observed for all groups but was particularly important in group 3 compared to the control group ($p < 0.05$) (**Figure 1**).

4.2. Testis Weight

The average testicular weights of rats in group 2 and group 3 were significantly higher compared to the control group. ($p < 0.05$). On the other hand, in group 4, it was significantly lower ($p < 0.05$). (**Figure 2**)

4.3. Testosterone Level

The mean testosterone level in the testis of the control group was 3.66 ng/L. We compared this level with the other groups and noticed no significant differences concerning groups 2 and 3. In group 4, the testosterone level was significantly lower compared to control ($p = 0.0051$). (**Table 2**)

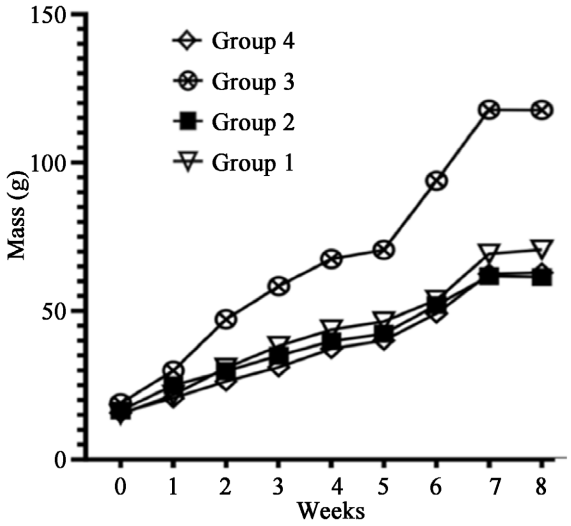

Figure 1. Evolution of body mass.

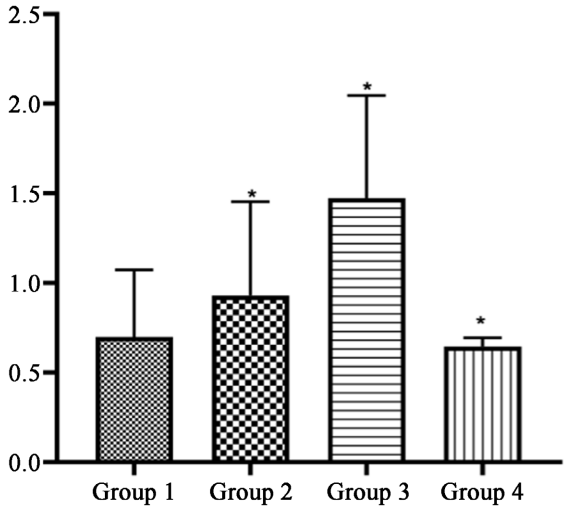

Figure 2. Testis weight in the different groups. * ($p < 0.05$ in comparison with control).

Table 2. Mean level of testosterone in the testis.

Group	Testosterone level
1 (Control group)	3.66 ± 0.0416
2	3.61 ± 0.0085
3	3.63 ± 0.0003
4	3.530 ± 0.0174**

**($p < 0.01$).

4.4. Histology

In Group 2, regarding the Johnsen score, there was no significant difference compared to the control (**Figure 3** and **Figure 4**). Respectively, we found a Johnsen score of 7.19 ± 1.62 for group 1 versus 7.32 ± 1.68 for group 2.

Concerning group 4, there were not late spermatids in most of the tubules (**Figure 3**). The Johnsen score was significantly decreased (**Figure 4**), with a mean of 5.96 ± 1.60. The best Johnsen score was observed in Group 3 (8.581 ± 1.12), the group that received the dose of 14 UI/kg corresponding to the administration of 100,000 IU of retinyl palmitate in the program of vitamin A supplementation.

5. Discussion

5.1. Excess of Vitamin A and Impaired Spermatogenesis

In our study, doses of 7000 IU/kg and 14,000 UI/kg of vitamin A were associated with an increased significant weight of the testicles compared to the control group. On the other hand, the rats in group 4 had a significantly reduced testicle weight compared to the control group.

At that dose of 28,000 IU/kg of vitamin A supplementation, corresponding to the dose of 200,000 IU given in the program, we noticed a lack of differentiation

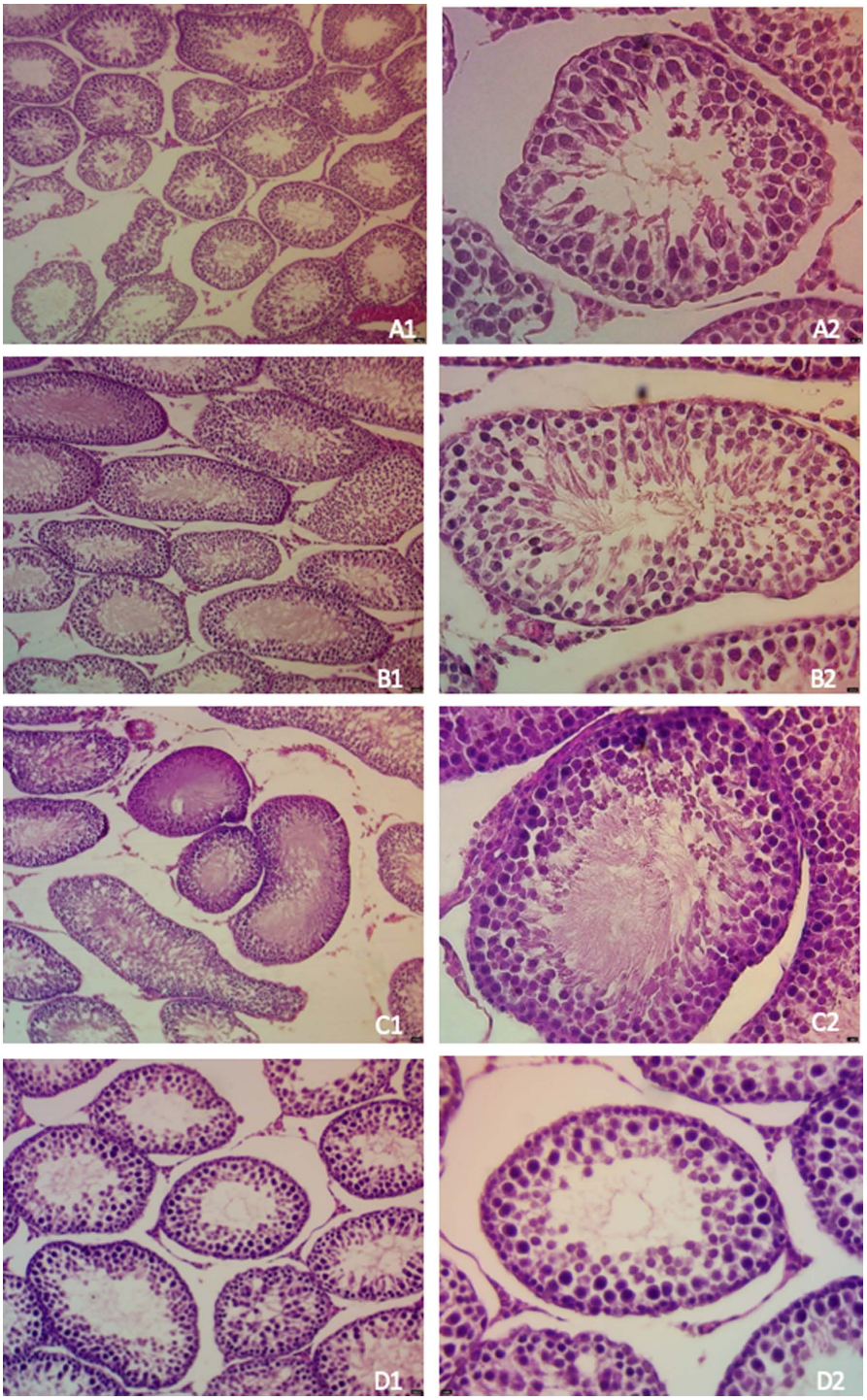

Figure 3. Effect of vitamin A supplementation on testicular histology among the different groups (H&E staining). A1 (×100), section of testis from group 1 (control group); A2 (×400), section of a seminiferous tubule from group 1, scored 7 (Johnsen score), with no late spermatids and few early spermatids. B1 (×100), section of testis from group 2; B (×400), section of a seminiferous tubule from group 2, scored 8 (Johnsen score), with few late spermatids. C1 (×100), a section of testis from group 3, and C2 (×400), a section of a seminiferous tubule from group 3, scored 8 (Johnsen score), with few late spermatids. D1 (×100), section of testis from group 4; D2 (×400), section of a seminiferous tubule from group 4, score 9 of Johnsen with many late spermatids, disorganized tubular epithelium.

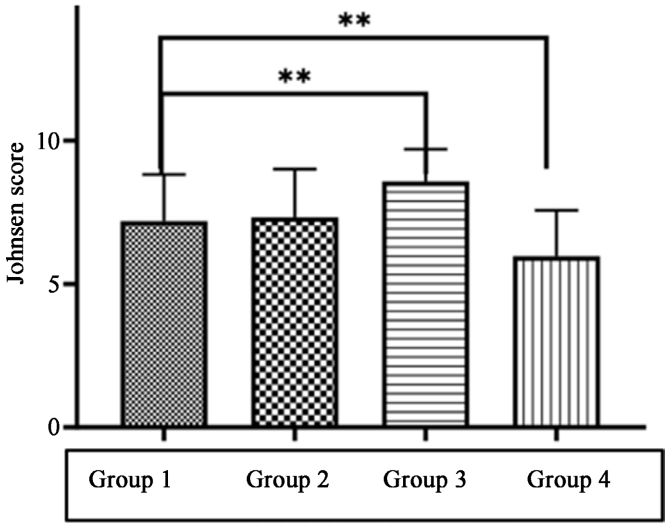

Figure 4. Classification of seminiferous tubules according to the Johnsen score (**p < 0.0001).

of round spermatids into elongated spermatids. To evaluate spermatogenesis, we used the Johnsen score for its clinical correlation with fertility. Thus, it is a classification used for rats [13], but also for human testis [14] [15]. A Johnsen score of 10 indicates maximum spermatogenesis activity, whereas a score of 1 indicates the complete absence of germ cells. The method is practical and easy to perform, providing a connection between the results of seminal analyses and those of testicular biopsies. [15]

Acute supplementation with a high dose of vitamin A (28,000 IU/kg) was associated with impaired spermatogenesis and a lack of differentiation into elongated spermatids. This finding was similar to the conclusions of Yokota *et al.*, [10], who worked on mice fed with an excess chronic dose of vitamin A since the perinatal period. But the difference is that, in our study, the supplementation consisted of a single acute dose of Vitamin A in the early life period, after birth (22 days).

The pathways determining various vitamin A conditions, such as low, moderate, or high retinol diet intake, determine germ cell fate in vivo, particularly in humans, is still a subject of research. [2] [10] [16].

5.2. Reversibility

Some studies have demonstrated that the intake of high replicate doses of vitamin A was able to reinitiate spermatogenesis among rodents after retinoic acid deficiency [17]. Unfortunately, in the case of hypervitaminosis, reversibility is not always obtained, and very few studies have been dedicated to the subject. To determine this reversibility, some authors allowed hypervitaminotic rats to recover for 50 or 100 days. After both recovery periods, they noticed that the focal lesions in the germinal epithelium were still unreversed. [18]. In Sadek *et al.*'s study, [19], there was a recovery 12 weeks after stopping the treatment, but this statement concerned male gerbils and not rats. It was found that 13-cis-retinoic

acid induced almost complete cessation of spermatogenesis and produced alterations in the cytoplasm of Leydig cells in adult males.

For men, studies are limited. Nevertheless, changes in semen parameters after systemic isotretinoin therapy are better documented. In a recent study, it was shown that systemic isotretinoin therapy negatively affects spermatozoa morphology [20].

In our study, despite a return to a normal diet, recovery didn't happen in the case of hypervitaminosis because our young rats presented at adult age with reduced testicular mass and incomplete spermatogenesis, with a decrease in the Johnsen score compared to the control.

5.3. Supplementation at a Young Age

Most studies focus on adult rats. In clinical practice, some authors, like Cirakoglu *et al.*, [21], underlined the controversy and lack of consensus about whether isotretinoin has any effects on spermatogenesis. His study concluded that both high-dose and low-dose isotretinoin disrupted spermatogenesis; however, it did not affect the hypothalamic-pituitary-gonadal axis. Nevertheless, very few studies are consecrated on phenomena occurring during the building testis architecture during the preprubertal or neonatal period and subsequently on the role of vitamin A at that early age.

Yokota *et al.*, [2] worked on 3-week-old mice fed with vitamin A excess for a period of 7 weeks. He compared his results with previous studies concerning rodents of 5 to 18 weeks old [10], fed with the same type of diet (excess of vitamin A), and noticed that the toxicity on spermatogenesis was more severe at a younger age than that at an older one in the long term.

The lack of studies concerning that period could be easily understandable if we consider that at an early age, spermatogenesis is "in building" before puberty. Nevertheless, Picut *et al.*, [22] insist on the necessity of histological examination of the testis of juvenile rats to characterize the safety of drugs for pediatric use. The author identified some time-sensitive points in development to be able to differentiate delayed development from direct toxicity and support further studies. Indeed, elongating spermatids should appear only between days 33 and 55 in the peripubertal. In early and late infanthood, spermatogenesis concerns, respectively, high proliferation of spermatogonia and mitotic activity wanes.

Each cell of the lineage possesses its own profile of RAR (retinoic acid receptor) ([16] [22]), and these receptors are involved in the regulation of spermatogenesis.

5.4. Regulation of Spermatogenesis and Vitamin A

Retinoids are involved in the regulation of testicular functions. Both excess and deficiency can block spermatogenesis. Thus, according to a recent study, FSH regulates retinoic acid signaling to commit spermatogonia into the differentiation pathway and meiosis [16]. In this review, the authors explored pathways at

the post-pubertal period and adult age in rodents. Indeed, the commitment of undifferentiated spermatogonia to differentiating spermatogonia and normal spermatogenesis requires the action of the gonadotropins FSH and LH. However, the molecular mechanisms by which FSH controls spermatogenesis via retinoic acid signaling need further investigation.

Recently, Lord *et al.* [23] demonstrated that the disposable number of spermatogonia and their quality would affect the initiation of spermatogenesis. Spermatogenesis would require retinoic acid (RA) to promote the induction of the undifferentiated to differentiating transition in transit amplifying (TA) progenitor spermatogonia. The author also describes how testicular architecture is critical for the mediation of retinoic acid responsiveness in undifferentiated spermatogonial subtypes in mice at adult age.

5.5. Blind Supplementation with Vitamin A

We are conscious of the need for supplementation in some areas of the world where vitamin A deficiency is one of the main causes of blindness. We understand that in response to the debate initiated by Mason *et al.*, [9] some authors warned by explaining that premature abandonment of the global vitamin A supplementation programs would not be prudent [24]. We agree with the advocacy of Benn *et al.* [25] in favor of rethinking the systematic supplementation of high doses of vitamin A. She proposed that a prudent phase-over was needed towards increasing frequent regular intakes of vitamin A at physiological levels, daily or weekly, replacing the high-dose periodic capsule distribution. Indeed, the impact of vitamin A deficiency on mortality was not confirmed by the most recent studies. Despite its lack of sensitivity and specificity, the determination of the concentration of retinol in the blood is used to assess vitamin A status. Serum retinol concentration determined by high-performance liquid chromatography (HPLC) is recommended by the World Health Organization to assess population vitamin A status [26]. This assay is expensive, technically demanding, and rarely available in developing countries [26]. An affordable and accessible technique should be available to assess vitamin A deficiency, and some authors have presented alternative methods [27]. So, Benn *et al.*, [25] explained that it remains questionable whether blanket, indiscriminate distribution of high-dose vitamin A to young children is advisable.

At the same time, in developed countries, the excess of vitamin A in the amount of fortified food and supplements responsible for overconsumption of vitamin A is becoming a public health problem. Safety for children should also be considered. According to the nutrition committee of the French Society of Pediatrics, intake of vitamin A often exceeds the recommendations [28].

6. Conclusion

Only a few publications are dedicated to the long-term effect of excess vitamin A on spermatogenesis. We observed a negative effect in the long term of a high

acute dose of retinyl palmitate supplementation at a young age on testicular development and differentiation. Despite a return to a normal diet after that supplementation, during childhood, impaired spermatogenesis was identified at adult age with an arrest of spermatogenesis.

Perspectives

More studies about the role of vitamin A in early life should be considered because of its long-term effect at adult age.

The reversibility of the defects observed in spermatogenesis after excessive intake of vitamin A is not clearly defined in the literature and needs further investigation.

Progress in molecular biology offers a new light in the understanding of the molecular pathways underlying the crucial role of retinoic acid in the endocrine regulation of spermatogenesis.

Limitation

The limitation of that work was that we cannot exclude that spermatogenesis alteration could begin in utero or in the early post-natal period before the beginning of the experimentation.

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Comment from the Authors

In order to comply with the comments of the reviewers, we have removed old bibliographic references that were not essential. However, we had to keep the strict essentials concerning princeps studies concerning the subject that were necessary to us to bring the novelties of our work (like the irreversible character of the detected alterations) or to report how the recent contributions of molecular biology allow to validate morphological observations, known for a very long time but explained recently (like the interaction "FSH-vitamin A-spermatogenesis").

Thank you for all the contributions you have made to make our paper better.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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