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Research Article

Comparison of the Level of Vitamins A, D and E of Plasma Seminal of Azoospermia and Normozoospermia

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Abstract

Background and Objective: Biological exploration of male infertility is important for its treatment. Seminal plasma, by its composition, presents numerous molecules that can be exploited in the investigation of new sperm biomarkers. The evaluation of new biomarkers of azoosperm seminal plasma aims to identify vitamins A, D and E which can serve as discriminating biochemical markers in the exploration of azoospermia. **Materials and Methods:** Thirty normozoospermic and 30 azoospermic sperm samples were collected by masturbation after three days of sexual abstinence from consulting patients at the Pasteur Institute of Côte d'Ivoire. After centrifugation of the sperm, the seminal plasma was collected and were analyzed for vitamin A, D and E. After extracting the vitamins from the seminal plasma, they were quantified using high-performance liquid chromatography. **Results:** The concentration of vitamin A in seminal plasma from normal samples was 1.66 ± 1.81 and 0.28 ± 0.52 mg/L in pathological samples. The average vitamin D concentration in seminal plasma of normospermia was 0.27 ± 0.40 and 0.08 ± 0.12 mg/L in seminal plasma of azoospermia. For vitamin E, the results obtained show an average concentration of 2.56 ± 3.58 mg/L in normal ejaculate and 0.33 ± 0.51 mg/L in pathological ejaculate. Only vitamins A and E showed a significant difference in the two categories of sperms. **Conclusion:** The determination of the concentration of vitamins A, D and E in seminal plasma showed that only vitamins A and E can serve as a biomarker for the differentiation of normozoospermic and azoospermic sperm.

Key words: Azoospermia, normozoospermia, seminal plasma, biomarker, vitamin

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Vitamins are essential for the growth and normal functioning of the body^{1,2}. They are associated with male fertility. They are linked to reproduction, spermatogenesis, androgen synthesis and secretion³. They are used in the treatment of certain infertilities and they are involved in the trapping of reactive oxygen species (ROS)^{4,5}. Vitamin A, for example, participates in the differentiation of the male genital tract, prostate and the seminal vesicles³. In sperm, a vitamin A deficiency leads to a reduction in the activity of acrosin and plasminogen activators, which are proteolytic enzymes essential for the induction of the acrosomal reaction. Vitamin A deficiency is also the cause of blocked spermatogenesis with a cessation of spermatogonia differentiation³. On the other hand, hypervitaminosis A induces testicular damage and disrupts spermatogenesis. Vitamin D has an indirect role in male fertility by regulating calcium levels in reproductive tissues, particularly the testicles⁶. Vitamin D receptors have been identified in Sertoli cells and sperm. Vitamin D and its receptors seem to play an important role in the survival of sperm and the acquisition of their fertilizing power. It is involved in the synthesis of certain hormones such as testosterone in the testicles⁶. Vitamin D deficiency is associated with a massive decline in fertility with incomplete spermatogenesis and developmental abnormalities. Vitamin E, also known as the anti-sterility vitamin, is a major antioxidant effective in protecting sperm¹. Several studies in animals and infertile men report a beneficial effect of vitamin E supplementation on sperm quality¹. The present study aims to evaluate the concentration of vitamins A, D and E in the seminal plasma of azoospermic sperm.

MATERIALS AND METHODS

Study area: The study took place in 2020 at the Pasteur Institute of Ivory Coast with in the Reproductive Biology Unit of the Department of Clinical and Fundamental Biochemistry, Côte d'Ivoire.

Materials

Biological material: This study involved the analysis of human sperm samples. They were collected from volunteer patients who came to the Pasteur Institute of Côte d'Ivoire for a biological exploration of fertility and who respected the three days abstinence period. The semen samples were 30 normozoospermia and 30 azoospermia seminal plasma.

Ethical consideration: The semen samples were collected with the written consent of the patients and the study was approved by the National Committee for Ethics in Life and Health Sciences (CNESVS), Order No. 036-13/CNESVS.

Methods

Spermogram: The spermogram was performed according to the instructions of the World Health Organization reference manual for semen analysis, fifth edition⁷.

Collection of seminal plasma: After cytological analysis of the sperm, the remaining sperm was centrifuged at 3000 rpm for 10 min. Seminal plasma was collected and stored at -20°C until the day of analysis.

Measured parameters: The parameters measured were the vitamins A, D and E concentration in seminal plasma.

Dosages of vitamins A, D and E: Preparation of standard stock solutions: The standard stock solutions of vitamins A and D were prepared at 10 mg/L after dilution in methanol. That of vitamin E was prepared at 20 mg/L. As for the internal standard; retinyl acetate, it was prepared at 1 mg/L. The HPLC calibration: Several successive dilutions of half of the solutions of the different standards were carried out to constitute dilution ranges. Thus, the dilution ranges for retinol and cholecalciferol were 1, 0.5, 0.25, 0.125 and 0.0625 mg/L. Those of α -tocopherol were 20, 10, 5, 2.5, 1.25 and 0.625 mg/L. These different dilutions were injected into the HPLC and repeated to obtain better calibration. The different concentrations obtained made it possible to draw the calibration lines.

Extraction of vitamins A, D and E was carried out using the cold liquid-liquid extraction process. To 500 μ L of retinol acetate, 500 μ L of seminal plasma was added. The mixture was stirred for 10 min and then 1000 μ L of cold ethanol was added. The whole was homogenized for 60 sec by the stirrer. Then, 2.5 mL of cold hexane was added to the preparation which was then homogenized for 5 min. The preparation was centrifuged at 3500 g for 15 min at 400°C. The upper hexanic layer was taken and evaporated under a flow of nitrogen, then 500 μ L of cold methanol was added to the pellet and the whole was stirred for 10 sec.

Chromatographic criteria: The experiments were carried out in isocratic mode. The mobile phase was composed of a methanol/water mixture in the proportions of 97 and 3%. The flow rate was maintained at 1 mL/min and the 55 detection spectrophotometry at 280 nm. The elution time was 10 min. The volume of the extract injected into the HPLC was 20 μ L.

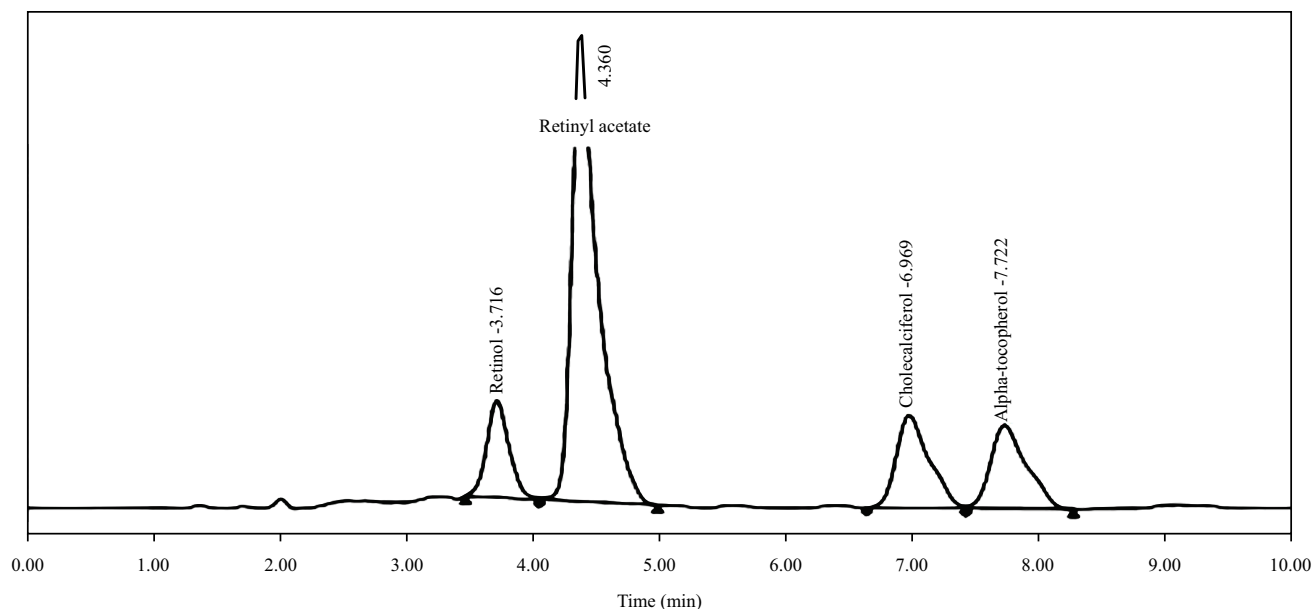
Statistical analysis: GraphPad Prism 9.0 software and Excel were used to carry out the statistical analyses. Results were reported as Mean $\pm$ Standard Deviation. Comparisons between pathological and normal seminal plasma were made using Student's t-test and Mann-Whitney tests for unpaired independent samples. A 95% confidence interval was used. A p-value < 0.05 is considered statistically significant.

RESULTS

The different parameters of the semen characteristics were shown in Table 1.

The results obtained confirm the presence of vitamins A, D and E in seminal plasma with respective

retention times of 3.716, 6.969 and 7.722 min (Fig. 1). Thus, the vitamin A concentration was 1.66 ± 1.81 mg/L in normospermia compared to an average concentration of 0.28 ± 0.52 mg/L in azoospermia (Fig. 2). The difference in vitamin A concentration of samples was significant (Table 2). The average concentration of vitamin D in seminal plasma of normal samples was 0.27 ± 0.40 mg/L (Fig. 3). This value was 0.08 ± 0.12 mg/L in abnormal seminal plasma. These values do not show any significant difference (Table 3). For vitamin E, the results obtained show an average concentration of 2.56 ± 3.58 mg/L in normal sperm compared to a concentration of 0.33 ± 0.51 mg/L in abnormal sperm (Fig. 4). These results present a significant difference (Table 4).



	Peak name	RT (min)	Area ($\mu V \cdot sec$)	Area (%)	Height (μV)	Height (%)	Amount
1	Retinol	3.716	13533	10.47	1110	13.09	10.000
2		4.380	77594	60.06	5355	63.14	
3	Cholecalciferol	6.969	19291	14.93	1061	12.51	1.000
4	Alpha-tocopherol	7.722	18782	14.54	955	11.26	20.000

	Units		Units
1	mg/L	3	mg/L
2		4	mg/L

Fig. 1: Chromatographic profile and retention time of vitamins A, D and E at 280 nm detection

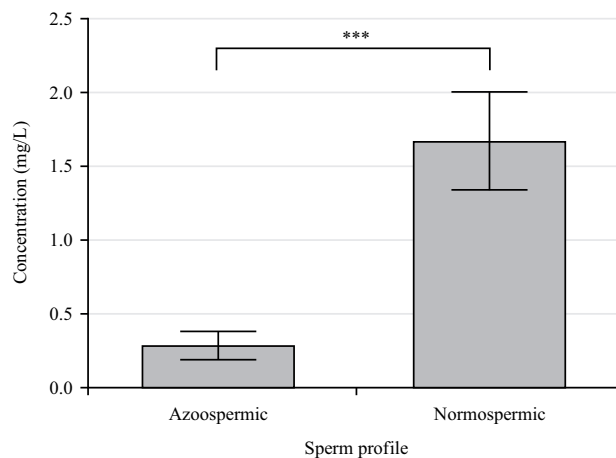


Fig. 2: Average concentration of vitamin A in normospermic and azoospermic seminal plasma

*Means present a significant difference ($p < 0.05$)

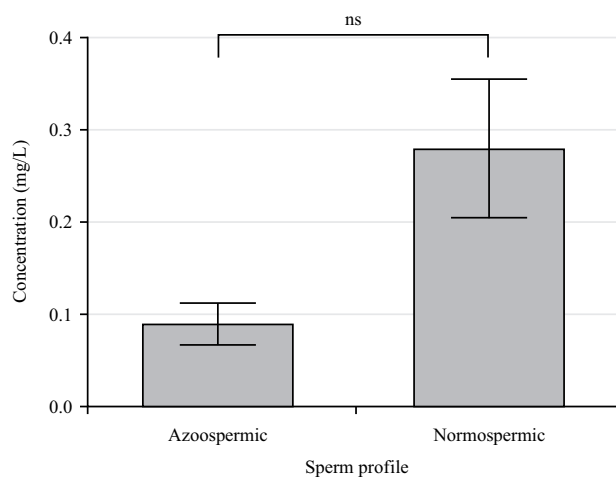


Fig. 3: Average concentration of vitamin D in normospermic and azoospermic seminal plasma

ns: No significant $p > 0.05$

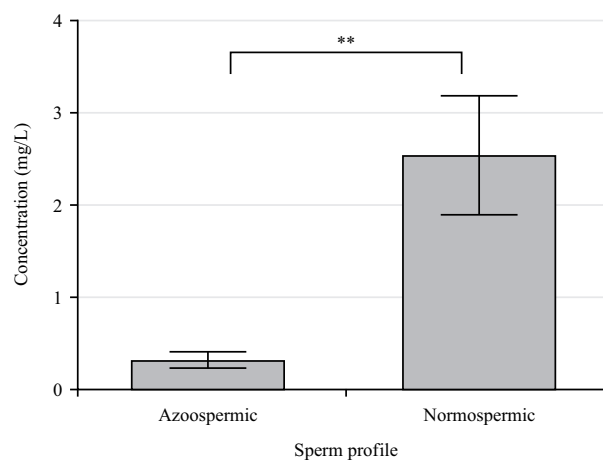


Fig. 4: Average vitamin E concentration in normospermic and azoospermic seminal plasma

*Difference is significant ($p < 0.05$)

Table 1: Azoospermic and normozoospermic semen characteristics

Characteristics of semen	Normozoospermic	Azoospermic
Number of sample	30	30
Volume (mL)	3.42±0.24	2.96±0.25
pH	7.58±0.05	7.61±0.04
Concentration (10 ⁶ /mL)	68.88±6.74	00±00
Mobility 1H (a+b) (%)	47.20±1.53	00±00
Mobility 4H (a+b) (%)	31.20±1.48	00±00
Morphology	23.52±1.82	00±00

Table 2: Statistical data of vitamin A concentration in seminal plasma

Concentration (mg/L)	Normospermic	Azoospermic
Maximum	6.350	1.779
Minimum	0.002	0.0020
Mean±Standard Error	1.668±1.819	0.2871±0.5201
p-value	0.0003	
Significance	Significant	

*Significant mean difference (p<0.05)

Table 3: Statistical data of vitamin D concentration in seminal plasma

Concentration (mg/L)	Normospermic	Azoospermic
Maximum	1.860	0.3950
Minimum	0.0010	0.0010
Mean±Standard Error	0.2792±0.4082	0.0894±0.1239
p-value	0.1947	
Significance	Not significant	

*Significant mean difference (p<0.05)

Table 4: Statistical data of vitamin D concentration in seminal plasma

Concentration (mg/L)	Normospermic	Azoospermic
Maximum	11.20	1.657
Minimum	0.0060	0.0080
Mean±Standard Error	2.561±3.589	0.3399±0.5148
p-value	0.0035	
Significance	Significant	

*Significant mean difference (p<0.05)

DISCUSSION

The difference in retinol concentration in azoospermic and normospermic seminal plasma was significant. This allows us to affirm that retinol can serve as a biochemical marker for the differentiation of normal and pathological sperm. This study was in agreement with that of Ghyasvand *et al.*⁸, who concluded that the concentration of retinol in the diagnosis and treatment of idiopathic infertility can be useful. Kand'ar *et al.*⁹ stated that there is a significant correlation between the concentration of retinol and that of sperm. The results of this study showed that the retinol concentration in the seminal plasma of normal sperm was higher than that of azoospermic ejaculate. Indeed, many authors noted that vitamin A deficiency affects male fertility³. Functioning of Leydig and Sertoli cells is regulated by vitamin A¹⁰. The vitamin A influences testosterone secretion in rodents and humans. It is also involved in spermatogenesis^{11,12}. This study also showed that in certain patients a low concentration of vitamin A in the

seminal plasma, with a normal spermogram reflects good functioning of the testicles.

The concentration of vitamin D in azoospermic seminal plasma was lower than that in normospermic samples and did not present a significant difference. This non-significant difference indicates that vitamin D cannot serve as a biomarker of differentiation between pathological and normal ejaculate. Some normal samples had low levels of vitamin D in their seminal plasma. Current results were in agreement with those of Boisen *et al.*¹³ who showed that in normospermic men, vitamin D serum levels are not correlated with sperm parameters and reproductive hormones (FSH, LH and testosterone), while sperm mobility showed a positive correlation with vitamin D. The vitamin D is a versatile signaling molecule with an established role in regulating calcium homeostasis. It plays an important role in male fertility¹⁴. Indeed, its receptors (VDR) and the enzymes metabolizing it are present in the gonads, the reproductive tract and on human spermatozoa¹⁵. Interestingly, expression

levels of vitamin D receptor and vitamin D inactivating enzyme CYP24A1 in human sperm serve as a positive predictive marker of sperm quality. Their expression is higher in the spermatozoa of normozoospermia than in infertile ones¹³.

The evaluation of the α -tocopherol content of the seminal plasma of azoospermic and normozoospermic sperm showed that the difference in concentration of these two groups is significant. It also showed that the vitamin E concentration of normozoospermic sperm was higher than abnormal samples. These results were in agreement with those of Bhardwaj *et al.*¹⁶, who mentioned that the level of vitamin E in the seminal plasma of azoospermia is low compared to normospermia. This study allows us to conclude that vitamin E can serve as a biomarker for the differentiation between pathological and normal ejaculate. On the other hand, Nouri *et al.*¹ showed that the vitamin E concentration of seminal plasma had a lower diagnostic value. However, Thérond *et al.*¹⁷ noted that there is a correlation between vitamin E concentration and sperm mobility. The results obtained during this study showed that the level of vitamin E in seminal plasma does not influence the normality of the spermogram. Indeed, some normozoospermic men had a low vitamin E content in seminal plasma. Also, it should be noted that α -tocopherol is the main lipid phase antioxidant compound of the vitamin E group. It is involved in the scavenging of reactive oxygen species². The presence of alpha-tocopherol in the aqueous phase of seminal plasma rather than in the sperm membranes where it is negligible, makes it more accessible to ascorbates which ensures its regeneration¹.

CONCLUSION

The determination of vitamins A, E and D in azoospermic and normozoospermic seminal plasma showed that vitamins A and E meet the biomarker criteria for differentiation of these two sperm categories. The concentration of vitamin D in the seminal plasma of azoosperms and normozoosperms did not have diagnostic value for azoospermia. In perspective, several research approaches emerge from this study. It will involve evaluating the concentration of vitamins A and E in the seminal plasma of different categories of pathological sperm to better define their impact on male infertility. It would also be essential to determine the precise role of the biomarkers identified in the diagnosis of secretory and excretory azoospermia.

SIGNIFICANCE STATEMENT

This study aims to highlight new essential biochemical markers of sperm for the diagnosis of azoospermia. It showed that the constituents of seminal plasma play an important role in the detection of certain sperm pathologies. Thus, the dosage of vitamins A and E showed a significant difference unlike vitamin D. Also, this study allows us to conclude that vitamins A and E can serve as biochemical markers of azoospermia. These results encourage us to further explore all categories of sperm pathologies to determine the character of biochemical markers of vitamins.

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