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Immuno-histochemical and bioinformatic study of the expression of alpha-foetoprotein at the epididymis in the rabbit «Oryctolagus cuniculus» supplemented by ascorbic acid

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Abstract

This study aimed to evaluate the effect of ascorbic acid (AA) (200 mg/Kg/b.w.) supplementation on the expression of alpha-fetoprotein (AFP) in the epididymis of male rabbits *Oryctolagus cuniculus*, as well as molecular docking between the AFP protein and vitamin C. For this, ten male rabbits were divided into two groups: control (T) and supplemented by vitamin C. The epididymis was dissected in order to conduct a histological study using haematoxylin eosin staining and Masson trichrome, immunohistochemistry by alpha-epididymis fetoprotein a protein expressed as molecular marker of cell proliferation (AFP), as well as molecular docking between the alpha-fetoprotein protein and vitamin C. Histological results revealed no structural changes in epididymis epithelium in AA-supplemented rabbits compared to control group. Regarding immunohistochemical examination by AFP, microscopic observation revealed negative immunolabeling in the control groups and supplemented with vitamin C. Docking was performed by the analysis software *Auto Dock Vina*. The molecular receptors were downloaded from the Protein Data Bank (PDB), and the vitamin C ligand was designed by MarvinSketch software and recorded as a 3D file. Our bioinformatic and molecular docking results reveal that the AFP gene in rabbits is located on chromosome 15 and indicate that is located on chromosome 15 in humans and demonstrates the types of mutation in the human AFP gene as nonsense substitution, AFP is primarily expressed during fetal development in both humans and rabbits, AFP levels are normally low but can increase in certain conditions such as pregnancy and certain liver diseases, including tumorigenesis and cancer development. There are several possible conformations for binding between vitamin C and alpha-fetoprotein, some of which are more beneficial and more stable than others. The first mode is the best mode with the most stable binding, indicating an affinity of -7.5 kcal/mols. In conclusion, our study demonstrates that alpha foetoprotein can associate ascorbic acid witch at a dose of 200mg/Kg b.w. has a beneficial effect on the tissular structure of the epididymis in male rabbit.

Keywords: Ascorbic acid, Alpha-fetoprotein, Epididymis, Rabbit, Histology, Immunohistochemistry, Bioinformatic, Molecular Docking.

Résumé

Cette étude visait à évaluer l'effet de la supplémentation en acide ascorbique (AA) (200 mg/Kg/ p.c.) sur l'expression de l'alpha-fEtoprotéine (AFP) dans l'épididyme de lapins mâles *Oryctolagus cuniculus*, ainsi que l'arrimage moléculaire entre la protéine AFP et la vitamine C. Pour cela, dix lapins mâles ont été divisés en deux groupes : le groupe témoin (T) et supplémenté en vitamine C. L'épididyme a été disséqué afin de mener une étude histologique utilisant la coloration de l'éosine hématoxyline et Masson trichrome, immunohistochimie par alpha-epididymis fetoprotein une protéine exprimée comme marqueur moléculaire de la prolifération cellulaire (AFP), ainsi que l'arrimage moléculaire entre la protéine alpha-fetoprotéine et la vitamine C. Les résultats histologiques n'ont révélé aucun changement structurel dans l'épithélium de l'épididyme dans AA-lapins supplémentés par rapport au groupe témoin. En ce qui concerne l'examen immuno-histochimique par AFP, l'observation microscopique a révélé un immuno-marquage négatif dans les groupes témoins et complété avec de la vitamine C. L'arrimage a été effectué par le logiciel d'analyse Auto Dock Vina. Les récepteurs moléculaires ont été téléchargés à partir de la banque de données de protéines (PDB), et le ligand de vitamine C a été conçu par le logiciel MarvinSketch et enregistré comme un fichier 3D. Nos résultats de bioinformatique et d'arrimage moléculaire révèlent que le gène AFP chez les lapins est situé sur le chromosome 15 et indiquent qu'il est situé sur le chromosome 15 chez l'homme et démontre les types de mutation dans le gène AFP humain comme substitution absurde, L'AFP est principalement exprimée pendant le développement fœtal chez les humains et les lapins, les niveaux d'AFP sont normalement faibles mais peuvent augmenter dans certaines conditions telles que la grossesse et certaines maladies du foie, y compris la tumorigenèse et le développement du cancer. Il existe plusieurs conformations possibles pour la liaison entre la vitamine C et l'alpha-ftoprotéine, dont certaines sont plus bénéfiques et plus stables que d'autres. Le premier mode est le meilleur avec la liaison la plus stable, indiquant une affinité de -7,5 kcal/mols. En conclusion, notre étude démontre que l'alpha fetoprotéine peut associer l'acide ascorbique sorcière à une dose de 200mg/Kg b.w. a un effet bénéfique sur la structure tissulaire de l'épididyme chez le lapin mâle.

Mots-clés : Acide ascorbique, Alpha-fetoprotéine, Epididyme, Lapin, Histologie, Immunohistochimie, Bioinformatique, Docking Moléculaire.

ملخص

تهدف هذه الدراسة إلى تقييم تأثير حمض الأسكوربيك (AA) (ملغ/كغ/ب200.) المكملات الغذائية على التعبير عن البروتين ألفا فيتوبروتين (AFP) في بربخ الأرانب الذكورOryctolagus cuniculus ، بالإضافة إلى الالتحام الجزيئي بين ذكور البروتين .VP تم تشريح الوبائيات من أجل إجراء دراسة نسيجية باستخدام تلطيخ يوزين الهيماتوكسيلين و Masson trichrome، الكيمياء الهيستولوجية المناعية بواسطة بروتين ألفا إيبيديميس فيتوبروتين معبر عنه كعلامة جزيئية لتكاثر الخلايا(AFP) ، بالإضافة إلى الالتحام الجزيئي بين بروتين ألفا فيتوبروتين وفيتامين سي. كشفت النتائج النسيجية عن عدم وجود تغييرات هيكلية في الوبائيات في الأرانب المكملة لـ AA مقارنة بالمجموعة الضابطة .فيما يتعلق بالفحص الكيميائي الهيستوكيميائي المناعي الذي أجرته وكالة فرانس برس، كشفت المراقبة المجهرية عن وجود علامة مناعية سلبية في مجموعات التحكم واستكملت بفيتامين سي. تم إجراء الإرساء بواسطة برنامج التحليل Auto Dock Vina.تم تنزيل المستقبلات الجزيئية من بنك بيانات البروتين(PDB) ، وتم تصميم رباط فيتامين سي بواسطة برنامج MarvinSketchوتم تسجيله كملف ثلاثي الأبعاد .تكشف نتائج الالتحام المعلوماتي الحيوي والجزيئي لدينا أن جين AFP في الأرانب يقع على الكروموسوم 15 وتشير إلى أنه موجود على الكروموسوم 15 في البشر ويوضح أنواع الطفرة في جين AFP البشري كبديل هراء يتم التعبير عن وكالة فرانس برس بشكل أساسي أثناء نمو الجنين في كل من البشر والأرانب، وعادة ما تكون مستويات وكالة فرانس برس منخفضة ولكنها يمكن أن تزداد في حالات معينة مثل الحمل وبعض أمراض الكبد، بما في ذلك تكوين الأورام وتطور السرطان. هناك العديد من التشكيلات المحتملة للربط بين فيتامين سي والبروتين ألفا فيتوبروتين، وبعضها أكثر فائدة واستقرارًا من البعض الآخر. الوضع الأول هو أفضل وضع مع الربط الأكثر استقرارًا، مما يشير إلى تقارب -7.5 سعرة حرارية/مول. في الختام، توضح دراستنا أن البروتين الجنيني ألفا يمكن أن يربط ساحرة حمض الأسكوربيك بجرعة من 200 .mg/Kg b.wله تأثير مفيد على التركيب النسيجي للبربخ في الأرنب الذكوري.

الكلمات الرئيسية: حمض الأسكوربيك، ألفا فيتوبروتين، إيبيديديم، أرنب، أنسجة، كيمياء مناعية، معلومات حيوية، رسو جزيئي.

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ABBREVIATIONS LIST

FSH: Follicle Stimulating Hormone

LH: Luteinizing Hormone

SPZ: Spermatozoa

AFP: Alpha foetoprotein

ROS: Reactive oxygen species

RNS: Reactive Nitrogen Species

AsA: Ascorbic acid

ADHA: L-dehydroascorbic acid

SVCT: Sodium-dependent vitamin C transporters

MD: Molecular docking

CADD: computer-aided drug design

PDB: Protein Data Bank

HE: Hematoxylin eosin staining

IHC: Immunohistochemistry

HRP: Horseradish Peroxidase

SUMMARY

INTRODUCTION

Alpha-fetoprotein (AFP) is a glycoprotein. It has traditionally been associated with fetal development and hepatocellular carcinoma in humans **(Desbene and Uversky, 2000)**. However, its presence and role outside these contexts, particularly in mammalian epididymis, have become of increasing interest because of potential implications for male reproductive physiology and pathology. In the rabbit *Oryctolagus cuniculus*, the epididymis plays a critical role in sperm maturation and storage, these processes are intricately regulated by various molecular factors **(Dacheux et Dacheux, 2014).**

In humans, the alpha-fetoprotein (AFP) gene is located on the long arm chromosome 4 (4q25).AFP is primarily produced by the fetal liver and yolk sac. Its initial role involves transporting essential fatty acids and regulating growth. it serves as a significant diagnostic marker in various pathological conditions, especially liver cancer , AFP is a protein expressed as molecular marker of cell proliferation **(Song et *al*., 1999)(Gillespie and Uversky, 2000).**

Vitamin C is known as L-ascorbic acid since it was observed as the factor needed for the treatment of scurvy (in Latin scorbutus, hence, "α-scorbutus"), the term also includes its oxidized form, L-dehydroascorbic acid, which can easily be converted into L-ascorbic acid in the human body **(Doseděl et *al*., 2021).**

Ascorbic acid (AA) is known for its antioxidant properties and essential role in collagen synthesis and hormone metabolism, is a key nutrient that serves as cofactor for numerous enzymatic reactions. AA must be obtained through dietary sources, especially fresh fruit and vegetables. AA supplementation may reduce oxidative stress and prevent several chronic conditions; few large, randomized clinical trials have tested it in patients with severe illness **(Berretta et *al*., 2020).**

Epididymis plays a crucial role in the maturation of sperm; their storage and their acquisition of functional abilities is essential to male fertility. Epithelial cells of the epididymis are involved in the secretion of various molecular factors necessary for these complex processes **(James et *al*., 2020).**

It has been shown that gene expression and protein synthesis are influence by our nutrition in various tissues, including the male reproductive system. The potential interaction between AFP and ascorbic acid in the epididymis remains unknown. This provides a unique opportunity to study their combined effects on spermatogenesis and sperm function.

Molecular docking is a computational procedure aimed at predicting the no covalent binding between macromolecules, typically a receptor and a small molecule ligand, efficiently utilizing their unbound structures, those derived from molecular docking simulations, or

homology modeling and the primary objective is to predict both the bound conformations and the binding affinity **(Trott and Olson, 2009).**

We are interested in making this work of memory which aims to evaluate the effect of ascorbic acid on the histology of the epididymis and immunohistochemistry of alpha-fetoprotein in male rabbits. On the other hand, the identification *in silico* by molecular docking of the association of vitamin C with a molecular marker of cell proliferation alpha-fetoprotein and determined the bioinformatics study of AFP gene.

In the literature review chapter, we present a bibliographic analysis describing the essential notions related to the global context of our study, including the target organ, “the epididymis”, and its link with vitamin C, as well as the use of cell proliferation mark, molecular docking and male genital tract, including its anatomy and a detailed study of the specific organ examined.

In the second chapter, we will expose the materials and methods, including the experimental setup and techniques used in the study, followed by presenting the results obtained, and concluding with future perspectives.

1. Male Rabbit Reproductive Tract

The reproductive system refers to all organs and structures that participate in the formation**,** maturation, and pressure release of the various components of sperm **(Alvarino, 1993)**.

The anatomy of the male rabbit's reproductive system shares many similarities with that of other mammals, yet it also exhibits distinct differences. During the embryonic phase, the genital tract undergoes differentiation; it develops from the primitive precursors of the tubules the male reproductive system is divided into different part (Figure 1a):

- The penis forms part of the external structure of the reproductive organ.
- Situated in the inguinal region, ventral to the anus, are the two scrotal pouches. Their primary function is to regulate the temperature of the testicles, maintaining it slightly lower than that of the body, aided by musculo-fibrous fibers.
- The scrotum also encompasses the two testicles the spermatic and testicular cords, as well as the epididymis **(Van Praag, 2015)**.

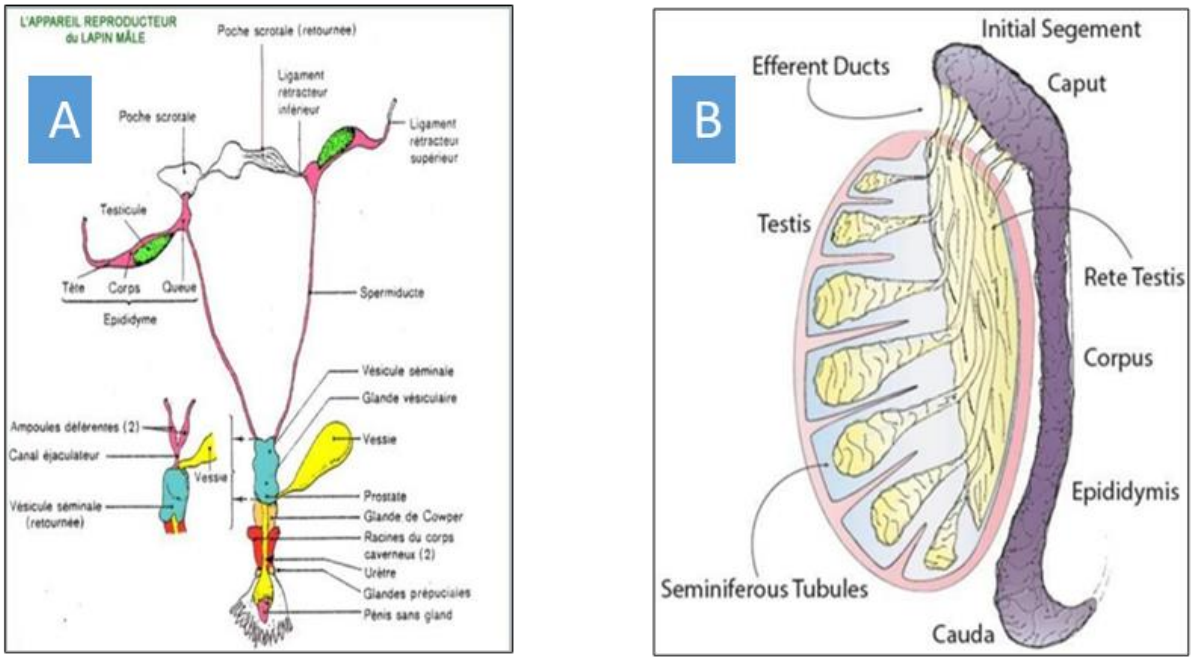


Figure 01: (a): Male genital tract **(Lebas et *al*., 1996); (b)** Testis and epididymis anatomy **(James et *al*., 2020).**

1.1.Epididymis

The epididymis in Greek epi for “on” and didumoi for “testis” **(Sullivan and Saez,**

2013). Epididymis is an organ attached to the testicle, formed of a very long tubule, strongly circumvented, which connects the testicle upstream via rete testis and efferent channels with the *vas deferens* downstream. This organ consists of a long tubule that connects the testis to the *vas deferens* and has four main anatomical regions **(Figure 1b)** each with unique characteristics and functions**:** the initial segment, caput (head), corpus (body) and cauda (tail) **(James et *al*., 2020):**

- A voluminous *caput*, it is the widest and shortest part of the epididymis. It is located at the upper end of the testicle and is hooked**.**
- *Corpus* representing the average portion is the longest part of the epididymis. It is formed of a sinuous duct that extends the entire length of the testicle.
- *Cauda* that forms a globular and mobile appendix. It is located at the lower end of the testicle and moves towards the *vas deferens.*

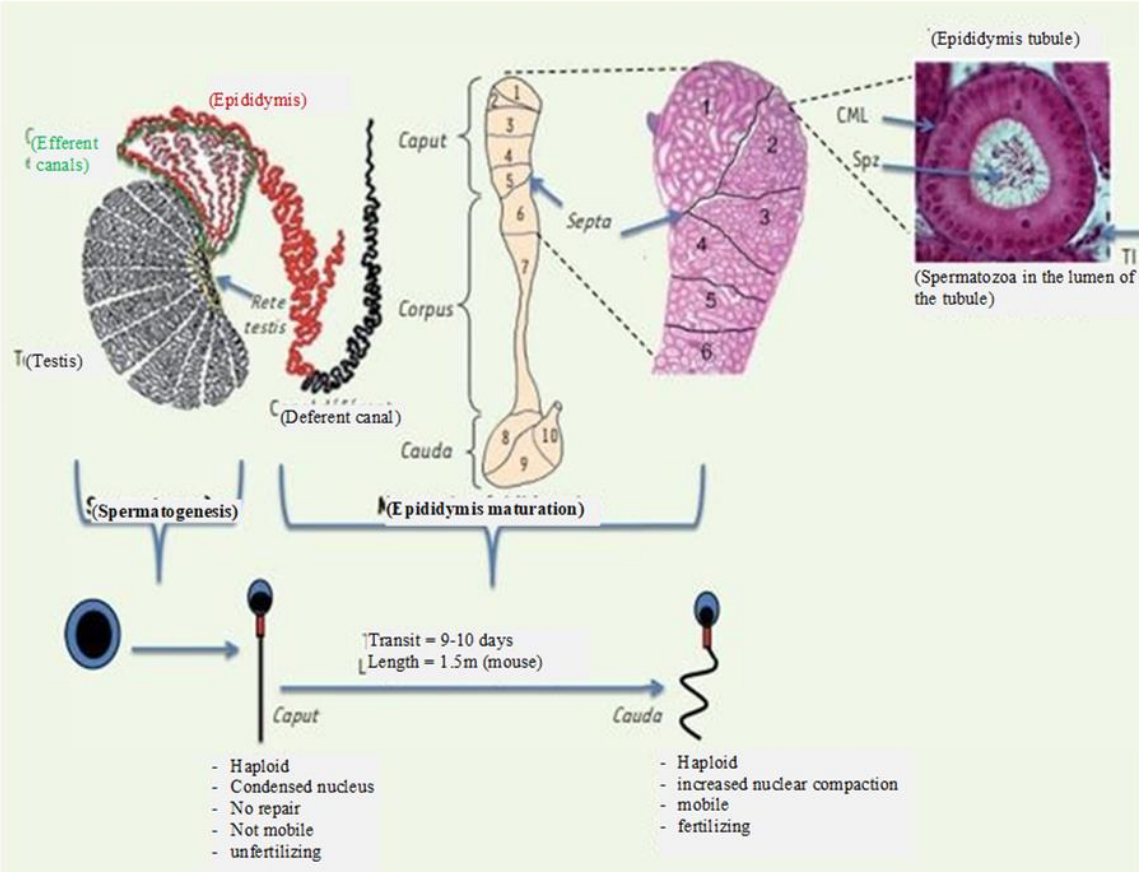


Figure 02: Mammalian epididymis **(Noblanc et *al*., 2012)**

They are subdivided into 10 segments separated by conjunctival septa. The pseudostratified epithelium of the epididymal tubule mainly functions by its diverse secretion

and reabsorption activities, which sequentially and locally modify the composition of the epididymal fluid that surrounds the gametes **(Noblanc et *al*., 2012).**

1.1.1. Epididymis Structure

It is important to understand the structure of the tubules that make up this organ. **There are six main cell types that make up the epididymal epithelium,** some of these cells are found within all regions of the epididymis while others are localized to specific regions,

Principal cells are the major cell type in the epididymal epithelium and exist along the entire epididymal duct, account for between 65 and 80% of the epididymal epithelium **(James and *al*., 2020).**

Apical cells are primarily located at the initial segment of the epididymal epithelium and also have endocytic activity,

Narrow cells also exist exclusively within the initial segment and are, as the name indicates, narrower than adjacent principal cells. These cells have been shown to secrete H+ ions into the epididymal lumen, and are responsible for endocytosis **(Cornwall, 2008).**

1.1.2. Epididymis physiology

Epididymis is a very important in the maturation and storage of sperm. The sperm produced in the seminiferous tubules are still immature and immobile when they arrive in the epididymis**.** During their transit through the epididymis, sperm acquire the ability to move and fertilize an egg cell **(cornwall, 2008).**

1.1.3. Epididymis histology

The epididymis is a coiled duct located on the posterior side of the testicle **(Gartner and Hiatt 2012)**. Its light is lined by a type of pseudo stratified epithelium composed of short basal and high main cells with stereocilia (long microvilli). The epithelium is separated by a basal membrane of the interstitial tissue wall that houses the smooth muscle cells. There are **7 cell types** present in the epididymal epithelium **(Table I)**: main cells, basal cells, halo cells, clear cells, apical cells, narrow cells and dendritic cells **(Robaire et *al*., 2006).**

Table I: Role of different cells constituted the epididymis epithelium

Cells	Function
Main cells	The main cells account for 65 to 80% of epididymal epithelial cells and are non-dividing in adults. In adulthood, the cells are prismatic and have basal nuclei and stereocilia, they synthesize a large number of proteins **(Robaire et *al*., 2006).**
Basal cells	The Basal cells are a small cell, round, elongated, and have a hemispheric appearance. Has attenuated processes that extend along the basal membrane from their main hemispherical cell body to cover a large part of the circumference of the epididymis **(Robaire et *al*., 2006).**
Halo cells	In the epididymal duct, halo cells form a narrow band of small cells with clear cytoplasm and a dense nucleus. They are identified as intraepithelial lymphocytes or monocytes that migrate into the epithelium forms an immunological barrier in the epididymis during post-natal development **(Robaire et *al*., 2006; Hoffer et *al*., 1973).**
Clear cells	The Clear cells are large prismatic cells found mainly in the body, tail and head of the epididymis, they are characterized by an apical region containing endosomes, multivesicular bodies, lysosomes, vesicles, as well as lipid droplets **(Robaire et *al*.,2006; Joseph et *al*., 2010).**
Apical cells	The apical cells are so called because their nuclei are located in the apical thirds of the cells they have a dense cytoplasm; these cells have ability to endocytose substances from the lumen of the cells, located ovoid nucleus and don't contact the basement membrane **(Robaire et *al*., 2006).**
Narrow cells	The upper portion of narrow cells is characterized by a bulging cytoplasm and an elongated nucleus. They are localized in specific segments, are few in number and are involved in acidification of the epididymal lumen **(Hermo et *al*., 2000).**
Dendritic cells	Dendritic cells possess immune cell markers that are involved in maintain immune homeostasis to protect spermatozoa **(Wang and Duan. 2016).**

1.1.4. Epididymis Functions

The epididymis has serval critical functions **(Table II)** in the male reproductive system including the maturation, storage and transport of spermatozoa and others **(Robaire et *al*., 2006).**

1.1.5. Endocrine control of epididymis functions

The epididymis is regulated by complex, multifactorial hormones **(Table III)** and neurotransmitters that involve coordinated interaction. Androgens, such as testosterone, are hormones that are vital for the development and maintenance of male reproductive function is an essential factor in male function plays a key role in epididymal physiology and is the principal regulators of gene expression

Table II: Epididymis functions

Function	References
Sperm Transport	The transport is achieved primarily by rhythmic contractions of the smooth muscle layers surrounding the epididymis. While contractions occur most frequently at the caput, they are most amplified at the cauda **(Elfgen et *al*., 2018).**
Sperm Concentration	Sperm concentration in the epididymis is necessary for increased sperm concentration in semen, an important factor in male fertility **(James et *al*., 2020).**
Sperm Protection	An additional function of the epididymis is to protect sperm cells during epididymal transit from damage caused by the external environment. Epididymal epithelial cells have high metabolic activity which results in the generation of reactive oxygen species that are harmful to sperm cells **(Hinton et *al*., 1996).**
Sperm Storage	Sperm storage cells are stored in the cauda epididymis before ejaculaton , in this location cells secrete factor that creat a specialised luminal environment which is keeping sperm in the resting state **(James et *al*., 2020).**
Sperm Maturation	The process of maturation occurs via direct contact of sperm with the contents of the epididymal lumen environment. Luminal environment is specific to each region of the epididymis and differences between regions are due to the varied cell composition of the epithelium and hormonal regulation, among other factors **(Cornwall, 2008).**

Table III: Hormonal regulation of epididymal

Hormones	Cells
Androgens	The epithelial cells of the epididymis express androgen receptors, the gene expression in the epididymis can be influenced by androgens, which has direct impact on the molecular processes involved in spermatozoa maturation, the receptor to androgens regulates the expression of specific genes of epididymis, contributing thus to the maturation of spermatozoid cells **(Britan and Drevet, 2006).**
Oestrogens	The stromal cells are responsible for controlling ion transport in the epididymis fluid. The epithelial cells of the epididymis express ion channels and transporters that actively regulate the ionic composition of the epididymis fluid, which is crucial for the survival, maturation, and function of the spermocytes; they are involved in the regulation of sperm concentration by promoting reabsorption of seminal fluid in the proximal region of the organ **(St-Pierre, 2002).**

1.1.6. Other hormones

Thyroid hormones (tri-iodothyronine [T3], thyroxin or tetraiiodothyronine [T4]) play an important role in epididymis functions, the action of these hormones, and in particular T3, is mediated by a nuclear receptor, the expression of which has been shown in rat epididymal cells **(Delrio et *al*., 2000).**

- FSH (follicle stimulating hormone): the role of FSH is mediated directly and/or indirectly, on the functions of epididymal epithelial cells and in particular in the maturation of SPZ, strongly altered in animal's mutant for the FSH receptor (FORKO) **(Menad, 2015).**
- LH can regulate important epididymal functions for SPZ maturation **(Menad, 2015).**

1.1.7. Epididymal secretion mode

Merocrine and apocrine drying modes **(Table IV)** are described as the mechanisms where epithelial cells release substances into the epididymal fluid in the epididymis.

Table IV: Epididymal mode secretion

MEROCRIN MODE	APOCRINE MODE
This mode of secretion occurs in most of the different cell types including the main cells of the epididymal epithelium. Proteins without membrane anchoring are released into the epididymis light **(Girouard, 2009).**	Apocrine secretion by the principal cells of the epididymal epithelium entails the formation of protruding apical blebs that extend into the epididymal lumen **(James et *al*., 2020).**

2. ALFA-FŒTOPROTÉINE (AFP)

AFP Is a serum glycoprotein; is a major protein of the embryonic plasma produced by both the yolk sack and fetal liver **(Gillespie and Uversky, 2000).** This glycoprotein is a member of the albuminoid superfamily, which also includes albumin, Gc globulin (vitamin D-binding protein), AFP is physiologically present in foetal serum. It has an important role during embryonic life, where it carries out various physiological activities, in particular as a transporter and cell growth factor **(Desbene and Gaillard, 2013).**

AFP plays a crucial role in the transport of various ligands such as fatty acids, hormones, minerals and bilirubin. AFP is involved in testicular tumours, since elevated levels can be detected in patients with testis cancer. Alpha-fetoprotein (AFP) is the "gold standard" biomarker for liver cancer; however, less known are the biological activities of AFP regarding carcinogenesis, growth, proliferation, and metastasis **(Mizejewski, 2016).**

2.1. Alpha-fetoprotein (AFP) structure

Alpha-fetoprotein (AFP) is a glycoprotein consisting of a single polypeptide chain with a molecular mass of around 69 kDa and consists of approximately 590 amino acids linked together by peptide bonds and is stabilized by a total of 15 disulfide (S-S) bridges.

The molecular weight and amino acid composition of AFP in humans, dogs, the rabbit and the rat share a similarity.

The protein has been divided into 3 domains, referred to as domains I, II, and III each consisting of 180 to 200 amino acids, with10 alpha helices with a loop configuration intermolecular dictated by disulfide bridging parts leading to a structural tertiary V-shaped. The domains I, II, and III have 4, 5, and 6 putative S-S bonds.

Even though there are similarities, there are significant distinctions between domains I, II, and III of AFP and serum albumin, suggesting that they possess distinct functions, such as the fatty acid-binding capacity unique to AFP, These structural variations may play a significant role in unlocking additional diagnostic or therapeutic uses for AFP, particularly if they interact with different molecules in biological systems.

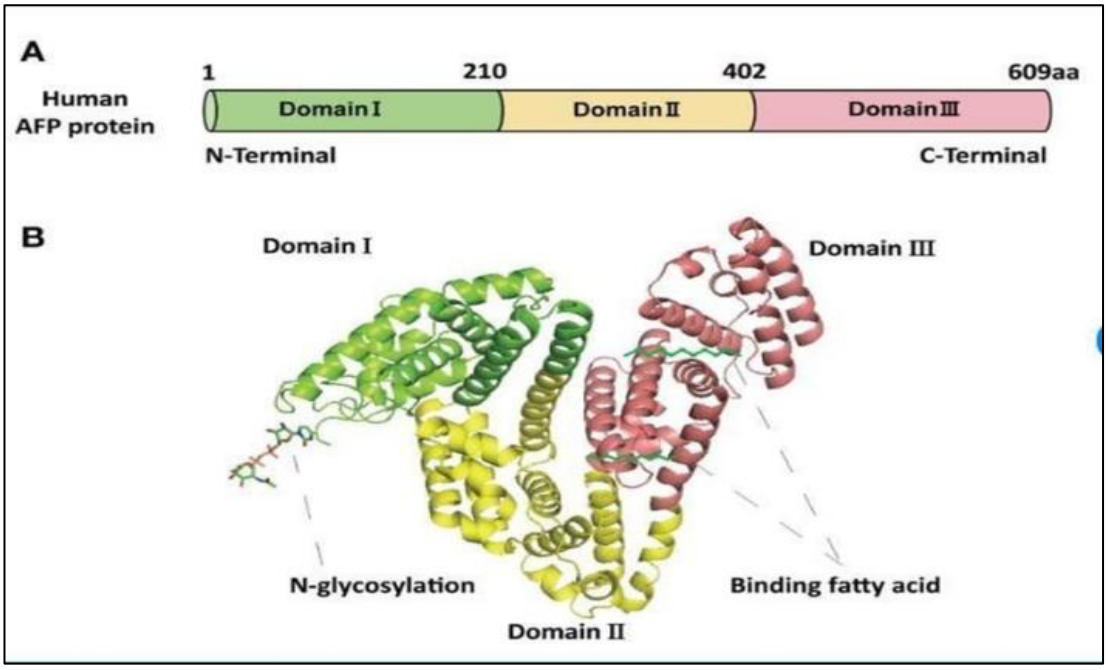


Figure 03: Basic structure of AFP. (A) The schematic diagram of the human AFP protein sequence with three domains. (B) The structure of AFP and the binding sites of AFP with fatty acids and N-glycosylation. The structure of AFP displays a V-shaped configuration, consisting of domain I (N-terminal) on the left side of the V, domain III (C- terminal) on the right side of the V, and domain II (middle region) at the base of the V **(Lü et al., 2024).**

The specific sequence of these amino acids determines the 3D structure and function of AFP Carbohydrate moieties: These are sugar molecules covalently bonded to the protein chain, and consisting of 4% carbohydrate residues and it can vary depending on the species.

2.2. AFP gene structure and products of its transcription

All the albumin family genes are located on the same chromosome. The AFP, SA, and α- albumin genes are positioned near each other and have a common direction of transcription. AFP genes are located on chromosome 15 of rabbit, and on the long arm of chromosome 4 of human (4q11-q13) **(Song et *al*., 1999).**

Structure of the regulatory region 5 -3 of the human AFP gene: five distinct regulatory elements that govern AFP expression are located upstream of the AFP transcription initiation site and include a specific tissue promoter of 250 bp, a repressive domain that is at least partially responsible for the decrease of AFP gene expression in the adult liver and three independent amplifiers located 2.5 kb, 5.0 kb and 6.5 kb upstream of the AFP promoter.

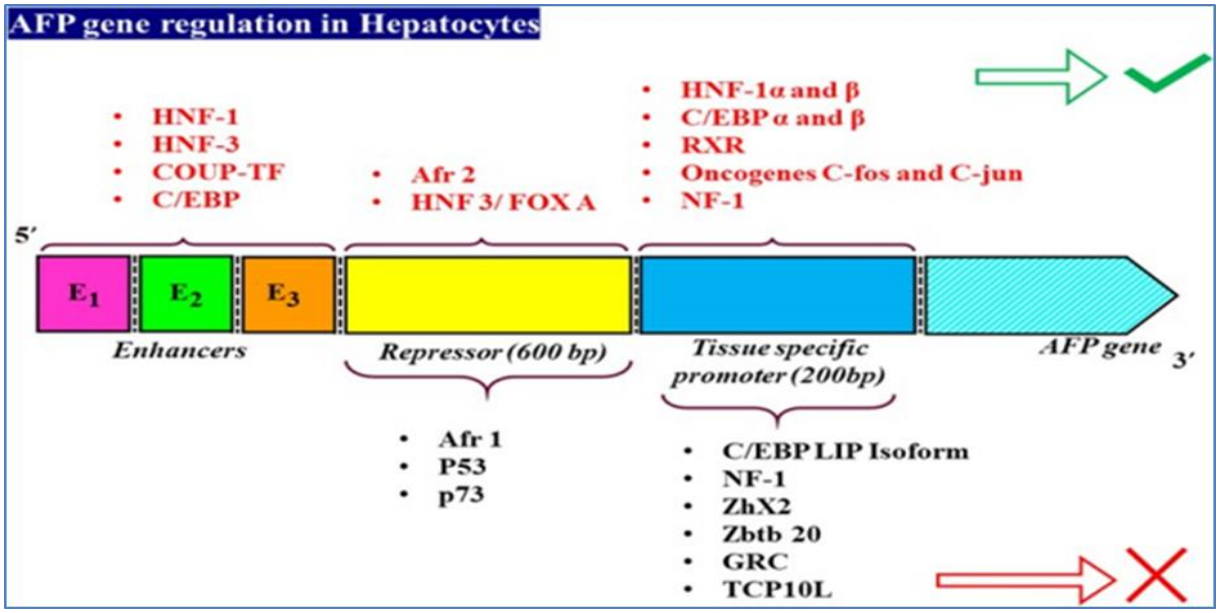


Figure 04: Human AFP gene 5′-3′regulatory region structure: Five distinct regulatory elements that govern AFP expression is located upstream of the AFP transcription start site and includes a 250-bp tissue- specific promoter, a repressor domain that is at least partially responsible for the decrease in AFP gene expression in the adult liver and three independent enhancers located at 2.5 kb, 5.0 kb, and 6.5 kb upstream of the AFP promoter. AFP gene activation: the transcription factors above the line are positive regulators of AFP gene transcription. Hepatocyte nuclear factor (HNF 1/3); Chicken ovalbumin upstream promoter-transcription factor (COUP-TF); C/EBP (CCAT- enhancer-binding proteins); Alpha-fetoprotein regulators (Afr1); Nuclear factor 1(NF-1); Zinc fingers and homeoboxes 2(Zhx2); Zinc finger and BTB domain-containing 20 (ZBTB20); Glucocorticoid receptor complex (GRC); Retinoid x receptor (RXR); T- complex protein 10A homolog 2 (TCP10L) **(Samban et *al*., 2023).**

AFP can be classified into three major isoforms, AFP-L1, AFP-L2, and AFP-L3, which vary in their affinity for Lens culinaris agglutinin. These isoforms are present in different amounts in different pathological situations. Two genetic loci Afr1 (raf) and Afr2 (Rif), that participate in regulation of AFP gene expression after birth.

It was shown that Afr1 can influence AFP transcript stability and/or it's processing. Perhaps an element of the AFP mRNA 3'-end secondary structure, a stem-loop which is highly conservative and is also found in human AFP mRNA, is involved in this process.

The AFP gene contains five elements located less than 7.6 kb upstream of the AFP transcription initiation site (+1) which defines the start of exon 1. The P promoter is from +1 to _250nt, the R is located between 250 and 838. The three distinct AFP activators were originally defined as fragments from -1.0 to -3.8, -3.8 to -5.3 and -5.3 to -7.6. Deletion analysis localized each enhancer into 200-300 bp elements called minimal enhancement region I MER I (black boxes), MERII (grey) and MERIII (hatched) respectively

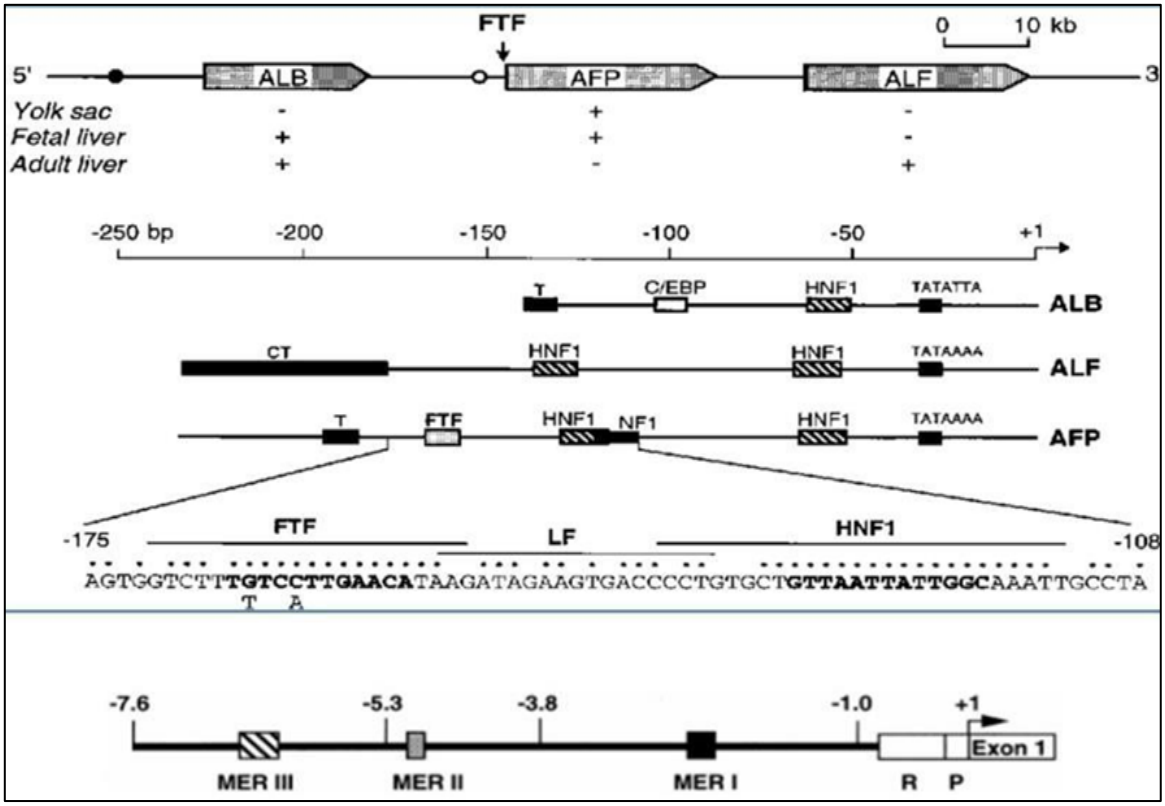


Figure 05: Structure of the AFP gene regulatory region

The mouse and rat AFP have three regulatory region distal enhancers, a promoter element and a silencer **(Liénard et *al.*, 2006):**

- **Promoter:** a sequence of 1000 base pairs (bp) of the AFP gene is characterized by tissue-specific promoter activity and contains multiple overlapping binding sites for ubiquitous and tissue-specific transcription factors **(St-pierre, 2009) these:**
- **Enhancers:** Like AFP promoter, the enhancers are tissue- specific and are not active in non-hepatic cells **(Hammer et *al*., 1987).**
- **Silencer:** These are DNA sequences that repress gene transcription by binding to transcriptional inhibitors. This reduced the activity of the AFP promoter.

3. OXIDATIVE STRESS

Oxidative stress is characterized by an imbalance between the production of reactive oxygen species (ROS) and the antioxidant network, with a bias towards the former **(Haleng et *al*., 2007).**

3.1. Reactive oxygen species (ROS)

Free radicals are chemical entities containing at least one unpaired electron in the outer shell which usually gives them high reactivity. The most frequently occurring free radicals and reactive molecules in biological systems are derived from oxygen (reactive oxygen species, ROS) and nitrogen (Reactive Nitrogen Species, RNS). ROS or RNS are formed during electron transfer reactions; by losing or accepting electron(s), ROS are important players in cellular proliferation, differentiation, migration, apoptosis, and necrosis **(Jomova et *al*., 2023).**

3.2. Antioxidants

Antioxidants are systems that have the ability to safely interact with free radicals and end the chain reaction before causing damage to vital molecules **(Oroian et Escriche, 2015)**. Antioxidants are created by the body as a defence mechanism against ROS. They can be enzymatic and no enzymatic. Vitamin C, vitamin E, selenium, zinc, beta carotene, carotene, taurine, hypotaurine, and glutathione are all nonenzymatic antioxidants. SOD, catalase, glutaredoxin, and glutathione reductase are including the enzymatic antioxidants **(Ahmadi et *al*., 2023)**. There are multiple mechanisms that contribute to antioxidant defence **(Losada-Barreiro and *al*., 2022)**:

- Delaying or inhibiting free radicals' production,
- Free radical scavenging,
- Changing free radicals into less toxic compounds,

- Delaying the formation of secondary toxic active species,

The antioxidant must be readily absorbed, eliminate RONs, and ultimately chelate metals at physiologically appropriate concentrations **(Losada-Barreiro and *al*., 2022)**. Antioxidants are classified into diverse classes: vitamins (vitamin C and vitamin E), carotenoids (carotenes and xanthophylls) and polyphenols (flavonoids, phenolic acids, lignans and stilbenes).

The biological properties of antioxidants, including flavonoids, phenolic acid, tannic acid, vitamin C (Ascorbic acid), and vitamin E, are multiple biological properties, such as anti-inflammatory, anti-carcinogenic and anti-atherosclerotic effects, reduce the incidence of coronary diseases **(Oroian et Escriche, 2015)**.

4. ASCORBIC ACID (AA)

Ascorbic acid, also known as acrobat or vitamin C, is a water-soluble antioxidant molecule that is fundamental for all living organisms **(Arabia et *al*., 2024)**. Serving as an antioxidant, ascorbic acid plays a central role in maintaining the vascular system. It aids in reducing atherogenesis in regulating collagen synthesis and facilitating the production of prostacyclin and nitric oxide **(Agwu et *al*., 2023).**

Gross formula	$C_6H_8O_6$
Purity	99,0%
Molecular mass	176,1241 g/mol
pH	2 – 3
Melting temperature	190° to 192°C
Mass density	1,65
Water-soluble 1g/3 ml (25°C)	
Soluble in alcohol 1g/40 ml (25°C)	

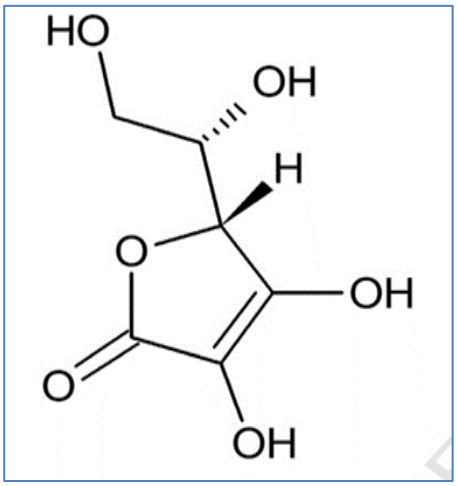


Figure 06: Propriety and Molecular structure of the ascorbic acid **(Caritá et *al*., 2020)**

4.1.Biochemical structure

The structure of ascorbic acid is related to that of sugar, have 6 carbon atoms: - 2 carbon function (carbons 4 and 5) and Two alcohol functions, - An enediol function (HO –C=C-OH) on carbons 2 and 3. Vitamin C is easily oxidized acid and destroyed by oxygen, alkali and high temperature **(Chambial et *al*., 2013).**

4.2. Physic-chemical property

The physicochemical properties of ascorbic acid are determined by its molecular structure **(Burtis, 1999).**

Vitamin C exists in two forms: L-ascorbic acid reduced form and L-dehydroascorbic acid (ADHA) oxidized form, which exist in reversible balance in tissues. This dynamic duo acts as a powerful catalyst for redox reactions in the body **(Lykkesfeldt et *al*., 2014).**

Chemically, vitamin C is an electron donor, or reducing agent, with all its physiological effects attributed to the transfer of electrons from acrobat.

Table V: Source and production ascorbic acid

Ascorbic acid production	Ascorbic acid source
Ascorbic acid production occurs on a significant industrial scale, with wheat or corn serving as the primary raw materials. Specialized enterprises process these grains into glucose via starch, which is then further converted into sorbitol. Through a series of stages, sorbitol is ultimately refined into the pure final product of ascorbic acid **(Ali and *al*., 2024).**	Animal sources are low in vitamin C content and typically around 30–40 mg/100 g. Vitamin C is identified in citrus fruits, green peppers, red peppers, strawberries, tomatoes, broccoli, Brussels sprouts, turnips, Indian gooseberry and other leafy vegetables. Therefore, plant sources become essential because of high content of vitamin C up to 5,000 mg/100 g. Absorption in the oral cavity occurs through passive diffusion, whereas in the gastrointestinal tract, it relies on active sodium-dependent vitamin C transporters (SVCT) **(Chambial et *al*., 2013).** The human body does not naturally produce vitamin C, It is vital to include this essential nutrient in the diet to support good health **(Schagen et *al*., 2012).**

4.3. Role of ascorbic acid

Its function as a free radical scavenger is now widely recognized as vital for preventing cancer, cardiovascular diseases, and cataracts **(Baron, 2009; Carr and Rowe, 2020; Smirnoff, 2018).** Also is cofactor for multiple enzymes essential for proper bodily function **(Blaschke et *al*., 2013; Young et *al*., 2015; Myllyharju, 2008).**

Enzymes essential for stabilizing the triple helical structure of collagen and can be used orally and topically for skin benefits **(Schagen et *al*., 2012).** Vitamin C amplifies the function of a broad array of iron (Fe2+) and α-ketoglutarate- dependent dioxygenases (α-KGDDs), including epigenetic regulators of DNA methylation, which are known to be crucial for maintaining genomic stability **(Brabson et *al*., 2021).**

Vitamin C increases telomerase activity and the expression of genes encoding telomerase-

related RNA and protein components that protect telomere stability **(Brabson et *al*., 2021).** Vitamin C has been explored experimentally in the treatment of male infertility, especially in cases involving nonspecific seminal infections. Research suggests that supplementing with vitamin C in men may enhance sperm quality. Additionally, in infertile women with luteal phase defects, vitamin C supplementation seems to raise progesterone levels **(Chambial et *al*., 2013).**

5. MOLECULAR DOCKING

The term "docking" generally refers to the interaction between a protein and a ligand **(Corbeil et *al*., 2007).** Molecular docking (MD) is a technique employed in computer-aided drug design (CADD) **(Zothantluanga et Chetia, 2022).** Molecular docking is a structure-based computational method that generates the binding mode and affinity between ligands and targets by predicting their interactions **(Muhammed et Aki-Yalcin, 2024).**

Usually, this involves a macromolecular receptor that has a defined three-dimensional structure and a small molecule called a ligand.

The primary goal of molecular docking is to determine the interaction mode of a complex composed of two or more molecules by exploring spatial orientations and identifying favorable conformations for ligand binding to receptors **(Corbeil et *al*., 2007).** Molecular docking has played a crucial role in bringing anti-HIV, anticancer, and various other drugs to the pharmaceutical market. There are numerous servers and programs used in molecular docking, each incorporating different force fields and algorithms for predicting poses, refining interactions, and generating target ligand interactions **(Muhammed et Aki-Yalcin, 2024).**

In this study, we used the **AutoDuck Vina program**, a novel program designed for molecular docking and virtual screening purposes. Vina uses a sophisticated gradient optimization method in its local optimization procedure. The calculation of the gradient effectively gives the optimization algorithm a sense of direction from a single evaluation **(Trott et Olson, 2009).**

5.1. Docking molecular Tools

5.1.1. Ligand

A ligand is a substance comprised of an atom, ion, or molecule possessing chemical functionalities that facilitate binding to one or more atoms or central ions. In biology, a ligand refers to a molecule that binds reversibly to a target macromolecule, such as a protein or

nucleic acid. Ligands typically play pivotal roles in functional structural stabilization, catalysis, modulation of enzymatic activity, and signal transmission. This structural alteration can consequently modulate its functional state and activity potential **(Leach, 2001).**

In this study the structure of ligands can be drawn using software MarvenSketch, which allows for precise design and visualization and saving of ligand structures in the 3D pdb format.

5.1.2. Receptors

For this study the structure of ligands can be obtained from the freely available three-dimensional structures stored in the Protein Data Bank (PDB). The PDB serves as a comprehensive repository of structural data for biological macromolecules such as proteins and nucleic acids. These structures are accessible at http://www.rcsb.org/pdb/ .

5.2. Docking stimulation

Consists of two essential steps:

- **Step 01 - Docking:** This initial phase entails placing the ligand into the active site of the protein and sampling different conformations, positions, and orientations (poses). Only the poses that exhibit the most favourable interactions are retained.
- **Step 02 - Scoring:** During this stage, the affinity between the ligand and the protein is assessed, and scores are assigned to the poses generated in the docking phase. This step aids in identifying the best pose among the options available.

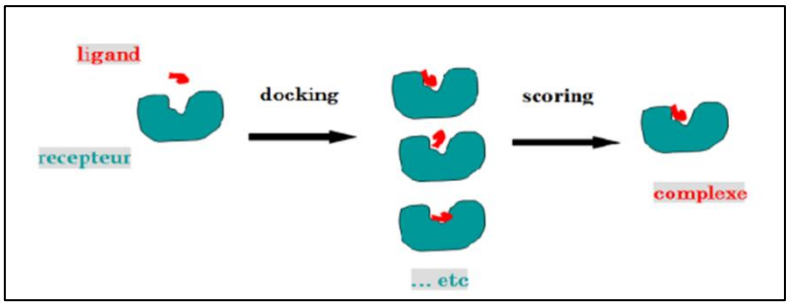


Figure 07: General Docking Protocol **(Ferey et *al.*, 2009)**

Presentation and location of the study

The objective of the study is to study the effects of vitamin supplementation (ascorbic acid) after sub-acute exposure (21 days) on the epididymis of male rabbits of the local strain «*Oryctolagus cuniculus*».The experiment was spread over the period from March to May 2024 divided into three periods :

- The first a practical internship at the experimental station-Saad Dahlab-Blida University 1 in which animal experimentation and animal sacrifice were carried out.
- The second was at the anatomy-pathology laboratory of the Beni Messous University Hospital -ALGER- over a period of 15 days. During this period, we performed haematoxylin-eosin and Masson trichrome stain, as well as immunohistochemistry:

  - ✓ Variations in epididymis weight.
  - ✓ Histological study of the epididymis (H&E stain and Masson's trichrome stain).
  - ✓ Alpha- fetoprotein immune histochemical (IHC) study.
  - ✓ This work was completed by a bioinformatics study on the alpha- fetoprotein protein.

1. Material

1.1. Biological material

1.1.1. Animal model

In this study, 10 male rabbits ITELV2006 (*Oryctolagus cuniculus*) obtained from the Technical Breeding Institute (ITELV, Baba-Ali), these rabbits were 4 months old and weighing between 2 and 3 Kg and they were transferred to the Rabbitry Unit of the Research Farm of the university BLIDA1.

The rabbits of our study:

- ✓ Rabbit pellets produced by SIM Sandres Algeria Spa
- ✓ water ad libitum
- ✓ housed in cages at 23 ± 2 C◦
- ✓ Humidity between 45% and 65%
- ✓ 12 h light/dark cycle throughout the experimentation period.

1.1.2. Taxonomy of the study rabbit

Systematics of the rabbit (Oryctolagus cuniculus) **(Wilson and Reeder, 2005):**

Kingdom	**Animalia**
Phylum	Chordata
Subbranch	Vertebrata
Class	Mammalia
Subclass	Theria
Subclass	Eutheria
Order	Lagomorpha
Family	Leporidae
Genus	Oryctolagus
Species	*Oryctolagus cuniculus*

Figure 08: Male rabbit *oryctolagus cuniculus* **(original photo)**

1.2. Non-biological material (ANNEXE)

2.1. Distribution of groups

Ten rabbits were randomly divided into two equal lots (groups) and treated by oral gavage for 21 consecutive days:

- ✓ Group 1: control and received 1 ml/day of distilled water.
- ✓ Group 2: received 1ml of ascorbic acid (AA) dilution (200 mg/kg bw).

2.2. Sacrifice and removal organs

On the 21st day of the experiment, the rabbits were weighed and sacrificed. After dissection, the testes are promptly excised, weighed, and the epididymis are dissected and placed in pill boxes containing 10% formol solution.

3. Histological study

The epididymis was placed in vials and fixed in a 10% formalin solution, which allows good fixation and penetration into the tissue.

3.1. Fixing the samples

The epididymis was cut into small fragments and deposited in plastic cassettes, then submitted to the histopathological study stages. The preparation of the blocks and the histological sections were carried out at the level of the pathological anatomy department (CHU Beni-Messous). Fragments of the epididymis were then put into 10% formalin (the most commonly used conventional fixator). It hardens the sample without damaging it. The samples are placed in special cassettes with perforated walls to allow the passage of liquids during the following manipulations.

3.2. Circulation

We use carousel type dehydration automaton, Step consisting of penetrating the paraffin into the fabric, as the paraffin is not miscible with water, and the samples must then be completely dehydrated before inclusion in the paraffin. The latter is also not soluble in alcohol used for dehydration, so there is a substitution by xylene.

3.2.1. Dehydration: Dehydration removes all water from the tissue by immersing the cassettes (containing the epididymis fragments) in three alcohol baths of increasing degree (70°, 90°, 100°) for ½ hour per bath.

3.2.2. Lightening: The cassettes are then emerged in xylene. Four xylene baths at a rate of 30 minutes per bath were carried out which serve to replace the alcohol in the tissues so that it is miscible in the paraffin.

3.2.3. Impregnation in paraffin: This is the last stage of circulation. Paraffin must be heated to liquefy in two successive baths of pure paraffin melted at 70°C to remove xylene. All operations are performed in a Leica TP102 PLC for a period of 12 hours.

3.3. Inclusion

The aim is to provide the fabric with an external support for the microtome cut and to ensure better preservation of the fabric afterwards. The inclusion medium most used is paraffin and automaton of inclusion. The inclusion was carried out according to the following steps:

✓ Paraffin wax was melted and maintained at a temperature of 60°C before being poured into metal moles.

✓ Orientation of fragments in paraffin (epididymis fragments are placed in the mold at a certain angle or position in order to see all the desired structures during microscopic examination).

✓ Place the cassettes over the moulds. After cooling, there is a hard paraffin block, inside which the fragment taken is included.

✓ The blocks are then unmoulded.

3.4. Performing Cuts

Paraffin cassettes containing epididymis samples are fixed on a microtome to make ribbon organ cuts at a thickness of 2 to 4 µm.

The resulting paraffin ribbons are spread over object blades and placed on a hot plate (45° to 60°) for 5 minutes.

The blades carrying the organ cuts are then dried in an oven at 100°C to allow for dewaxing.

3.5. Histological HE staining

The following steps are necessary:

3.5.1. dewaxing: to remove paraffin from the fabric and thus allow the dye to penetrate, the blades are passed through three xylene baths for 3 to 5 minutes per bath.

3.5.2. Hydration: to remove xylene from the tissue and replace with water. The agent used is alcohol; the blades are passed in 3 baths of 2 minutes each at decreasing concentrations 100°, 90°and 70°. Then the blades are rinsed in running water for 3 to 5 minutes and then we move to coloring.

Hematoxylin-eosin staining is a staining technique used in pathological anatomy and histology, it consists of two dyes: the first is haematoxylin (colors the acidic components of the tissue like the nucleus), and the second is eosin (colors basic tissue components like cytoplasm); Staining goes through the following steps:

✓ A Harris haematoxylin bath (for 6 minutes) to stain blue.

✓ Rinsing with tap water for washing blades.

✓ Coloring with eosin (for 1 minute) to color pink.

✓ Rinse with tap water to clean it from the blades.

✓ After coloring, drying in the oven.

3.5.3. Mounting the Blades

Mounting is the last step before microscopic reading. The coverslips are applied to the slides using Eukitt, which promotes adhesion of the coverslip to the slide. This step offers protection against discoloration caused by oxidation by ambient air and the fumes of certain chemicals. After mounting, the slides are dried on absorbent paper and cleaned with xylene, ready for microscopic observation.

3.6. Masson's trichrome stain

Masson's trichrome staining is a staining technique used histology, it consists of three dyes:

- ✓ The first is Mayer hemalun color of the nucleus in dark blue core in black.
- ✓ The second is a precise blend of acid fuchsine and ponceau red color the cytoplasm in red.
- ✓ The third color is the green light colors the collagen in green.

3.6.1. Staining goes through the following steps

- ➢ The blocks are fixed on a microtome to make ribbon organ cuts at thickness of 10 à 12 µm.
- ➢ Dewax in three successive xylene baths, leaving for three minutes per bath.
- ➢ Rehydrate in water for four minutes.
- ➢ Place in Mayer's hemalun for at least 10 minutes.
- ➢ Rinse with running water, running the blade under water on the opposite side of the mixture to avoid peeling off the cut, then soak in tap water for four minutes (this step is essential for hemalun differentiation; differentiation can also be achieved in a buffered salt phosphate solution).
- ➢ Color in fuchsin-culvert for 5 minutes.
- ➢ Perform a quick rinse in two successive baths of 1% acetic water, or simply by passing under water on the opposite side of the preparation.
- ➢ Mordancer in 1% phosphomolybdic acid bath for about ten minutes.
- ➢ Do not rinse after etching.
- ➢ Then color for twenty minutes in light green.
- ➢ Rinse in two successive baths of 1% acetic bid stilled water, leaving for five minutes for each bath.

3.6.2. Mounting the Blades

The blades are set aside and strips bearing eukitt are placed on them to protect the samples. Then they are left in the open air to dry.

4. Immune histochemical study (IHC)

Immunohistochemistry (IHC) is a technique used to precisely exact the position and distribution and visualize the presence of the protein of interest within the analyzed tissue section. While it may not offer the same quantitative sensitivity as immunoassays like Western blotting or ELISA, IHC allows for the examination of biological processes within the context of intact tissue. This feature proves particularly valuable in the evaluation of disease progression and treatment, notably in cancer research. Overall, the insights gleaned from IHC, when coupled with microscopy, offer a comprehensive understanding that complements data obtained through alternative methodologies.

4.1. Principle

Immunohistochemistry is based on the affinity antigens and antibodies; the method entails the detection of an antigen through the initial binding of a primary antibody, followed by a secondary antibody coupled to an inert polymer carrying enzymes. Subsequently, this complex is visualized using a substrate that undergoes hydrolysis, resulting in the formation of a brown precipitate. Structures that have bound the primary antibody become easily distinguishable due to this staining. Counterstaining with haematoxylin is then applied, imparting a blue color to nuclei and cytoplasm. This dual staining enables the visualization of all structures, thereby facilitating the microscopic identification of areas of interest.

Antibodies are pivotal reagents in immune histochemical reactions: without suitable antibodies, there can be no immunoreaction.

- The primary antibodies are two types: Monoclonal (mice) recognizing a unique epitope and polyclonal antibodies recognizing multiple epitopes of the antigen (mice, rabbit)
- The secondly antibodies use of anti-Ig antibodies from the rabbit or mice linked with the enzyme’s peroxides or alkaline phosphatase.

In this study use a specific automaton for IHC.

Figure 09: IHC automat technic **(Original photo)**

4.2. Steps of immunohistochemistry (IHC)

- **Step 1: Sample Preparation**
  - Attaching the tissue taken
  - Dehydration of the tissue
  - Paraffin inclusion
  - Cut the tissue at microtome 1 to 2µm
  - Drying blades in oven at 54° for 24 hours for enhanced tissue adherence to slides
- **Step 2: marking**
  - Unpacking: immersion in 4 baths of xylene for 5 minutes each.
  - Rehydration: submersion through 5 minutes each in 3 baths alcohol of 100%, 95%, and 70%.
  - Washing with tap water continued by distilled water for 5 minutes.
- **Removal of epitopes**: Aid expose epitopes that are naturally masked or due to fixing, guarantee antibody-antigen binding **(Shi et *al*., 2001),** a use is done in a water bath for 40 minutes at a temperature of 98 °C in a citrate buffer, breaking the bonds between proteins during fixation and facilitating the passage of antibody.
- **Blocking non-specific sites (and endogenous biotins if necessary):** Prepared Five percent bovine serum albumin (BSA) by Dissolving 0.25 g BSA (A 9647, Sigma-Aldrich) in 5 ml Permeabilization solution (pH 7.4). This solution will be utilized to block non-specific binding and has to be prepared freshly and stored at 4°C for use within 48 h. It can, in addition, be diluted to 1% in order to be utilized for primary and secondary antibody dilutions.

- **Primary antibodies:** Put some drops of the antibody AFP on the circled part of the tissue on the blade. Cover the blades and incubate for 30 minutes. After, for 5 minutes rinse in 2 TBS buffer baths.

- **Step 3 : Revelation**

✓ Secondary antibodies HRP or AP/ Polymer HRP or AP: for amplification of the signal conjugated to the enzyme HRP (horseradish peroxidase) with secondary antibodies diluted to 1%, this solution, were deposited on the slide then incubated for 30 min.

✓ Incubated for 30 min.

✓ chromogen (DAB, AEC, Permanent Red …) : in the first prepare with a solution of 1 ml of buffer substrates containing the peroxidase enzyme and a drop of DAB containing a chromophore this preparation applied on the slides and then for the demonstration of the existence of a reaction between the antibody and the antigene that manifests itself in the appearance of a brown color, Incubate the blades for 5-8 min

✓ After rinsed for 5 min each in 3 PBS trays

✓ Haematoxylin counter-staining: To color the cores blue-violet

✓ Dip the blades in Mayer haematoxylin (5 min).

✓ Rinse with water to weed out excess haematoxylin.

✓ Then the blades were installed in an ammonia bath to remove the overload blue dye.

✓ Continued by a water rinse.

- **Step 4: Blade mounting**: use the Eukit to attach the slats to the blades

- **Step5: Microscopic observation, reading and interpretation:** use the photonic microscope for observation, take the images in x40 and transformed the slides to QuPath software V 0.3.2. Cells with dark brown cytoplasm and nucleus were considered as positive cells. For Protein expressions of AFP in epididymis tissue, use the statistically analyzed

5. Statistical study

The statistical analyses were conducted using Statistica version 10.0 (Stat Soft Inc., Tulsa, Oklahoma, USA). Various parameters were analyzed and presented as mean ± standard deviation (SD). To compare the means of each parameter among the different batches studied, we employed one-way analysis of variance (ANOVA), followed by Duncan's post-hoc test. A p-value < 0.05 was considered statistically significant.

6. Docking protocol

Molecular docking is done using Auto Dock Vina software

6.1. Receptor preparation

- Molecular receptors are downloaded from **the Protein Data Bank (PDB) (http://www.rcsb.org).**
- Crystal structures of the AFP binding protein targeted as a receptor.
- AFP is prepared via the protein visualization Autodocktools version 1.5.6 programs (The Scripps Research Institute, USA).
- by eliminating water molecules and previous heteroatoms.
- Protein.pdbqt is prepared by defining polar hydrogens and Kollman fillers on AutoDock 1.5.6 tools to be ligand-receptive.

6.2. Ligand preparation

- The Carvacrol ligand is designed by the **MarvenSketch software**.
- Saved as 3D.dbp files.
- Prepared as ligand.pdbqt for docking simulation by defining possible torsions to the torsion shaft.
- Rotating and non-rotating links via Autodocktools version 1.5.6 programs (The Scripps Research Institute, USA).

6.3. Simulation

- A cubic grid box is defined on the active site and saved as config.txt.
- The empirical algorithm **Auto Dock Vina** generated potential and notation functions based on the force field to guide the choice of ligand poses and correct compound/protein structure combinations.

6.4. Analysis

Predictions of ligand placement and cluster notation are interpreted and visualized using discovery studio software 0.4. Then 2D/3D representative structures are used for the visualization/interpretation of amino acids/binder ligand pocket.

6.5. Result

Docking scores presented as link energy (ΔG), mean square deviation RMSD (Å), inhibition constant (Ki), interaction types and distances (Å) were considered as inputs to discuss interaction results.

7. Bioinformatics study of alpha-fetoprotein

The goal of this study and determines the localization gene alpha-fetoprotein in the rabbit and mutation types in the human and also determine the 3D structure of alpha-fetoprotein

7.1. NCBI

NCBI conducts research in software biologyit is a database that integrates information specific to genes to from multiple data sources (https://www.ncbi.nlm.nihngov/).

7.2. SWISS-MODEL

Was a developer in the domain of automated modeling as the first protein modeling service on the internet. In combination with the Swiss-PdbViewer visualization tool, the Internet-based workspace and the SWISS-MODEL repository, it provides a fully integrated sequence for structuring the analysis and modelling platform (www.swissmodel.expass.ord).

7.3. COSMIC

The Catalogue of Somatic Mutations in Cancer – is the world's largest source of expert manually structured somatic mutation information related to human cancers. This extensive database compiles detailed information on somatic mutations, which are genetic alterations accumulated by cells that can principal to cancer. The resource is invaluable for cancer research, offering insights into the genetic basis of cancer, aiding in the identification of potential therapeutic targets, and supporting the development of personalized medicine (COSMIC | Catalogue of Somatic Mutations in Cancer (sanger.ac.uk)).

RESULTS AND DISCUSSION

Our work focuses on the histological variations of epididymis and immune-histochemicals of the expression of the protein alpha-fetoprotein at the epididymis level in rabbits of local strain treated by ascorbic acid, while demonstrating the ameliorative effect of vitamin C supplementation.

Our results below include:

- The rabbit's epididymis weight.
- Histological study of the epididymis in control rabbits compared with rabbits treated with ascorbic acid.
- Immune histochemical (IHC) study of alpha-fetoprotein protein expression in both groups.
- Molecular docking of AFP
- Bioinformatics study of the AFP gene

1. Variation in body weight during the experimental period

During the 21 day study period, no deaths occurred in any of the experimental groups. Additionally, there was a consistent increase in body weight observed in both the control group and the AA-supplemented groups. This suggests that the administration of AA did not lead to any deaths and did not negatively impact the subjects' body weight, thus reinforcing the positive aspect of this intervention within the study.

	Control	Ascorbic acid
Initial BW	2.81 ± 0.01^{a}	2.86 ± 0.02^{a}
Final BW	3.14 ± 0.03^{a}	3.17 ± 0.02^{a}

Table VI: Animal's initial and final body weigh

2. Results of the histological study of epididymis in rabbits

2.1. Histology of epididymis in control rabbits' colors by HE staining

Microscopic observation of histological sections of the epididymis at low magnification (at Gx100) and high magnification (b Gx400) in the control rabbit after hematoxylin-eosin staining reveals the structure of the epithelium and epididymal tubes.

The epididymal tube (TE) is a long, contoured tubule, surrounded by interstitial tissue (TI) and a thin layer of smooth muscle (MS). Some parts of the tube contain mature sperm (Spz). The pseudostratified epithelium (Ep) is composed of two types of cells: the main cells (MC), which are large cylindrical epithelia and are lined with immobile stereomies (Scils), and the basal cells (BC), which are small and spherical, and located near the base of the epithelium.

2.2. Histology of epididymis in rabbits treated by ascorbic acid and colors by HE coloring

The plates 2 represents the histological sections of epididymis in rabbits treated for 21 days with ascorbic acid at low (a: Gx100) and high (b: Gx400) magnification after haematoxylin-eosin staining exhibited no structural modifications across the different regions of the epididymis:

- The epididymis tubes (ET) have a regular contour.
- The appearance of the Stereocilia (Scils).
- The appearance of the vacuols around the cells.
- An increase in diameter of the intertubular space and light of the tubes epididymis.
- The appearance of sperm clusters (Spz).

2.3. Histology of epididymis in controls rabbits colors by Masson trichrome coloring

The plates 03 represents the histological sections of epididymis in rabbits at low (a: Gx100) and strong (b: Gx400) magnification after Masson trichrome coloring showed several changes in the different epididymis structures:

- The epididymis epithelium (Ep) is an increase the splicer and colored in red.
- The muscle fibres are stained in blue, and degeneration of vacuoles.
- The appearance of small cells debris (Dc) in the lumen (EL) of the epididymis tube (TE).
- The epididymis tubule (ET) has a regular contour.
- The appearance of spermatozoa clusters (spz).

2.4. Histology of epididymis in rabbits treated by ascorbic acid and colors by Masson trichrome coloring

As shown in Plate 4, histological sections of the epididymis in rabbits treated by Acid ascorbic coloring by Masson Trichrom (A: Gx100, B: Gx400) present a regularly contoured epididymis tubule (ET) , an increase in tubular lumen diameter , also the degeneration of sperm clusters in the epididymis epithelium , the light level becomes clear with degeneration of accumulation of cell debris (Dc) in the epididymis light level , also decreases in epididymis epithelium leads to an increase in the light level , the scils have almost disappeared , the appearance of collagen fibres with green color.

Histology of epididymis in control rabbits (HE coloring)

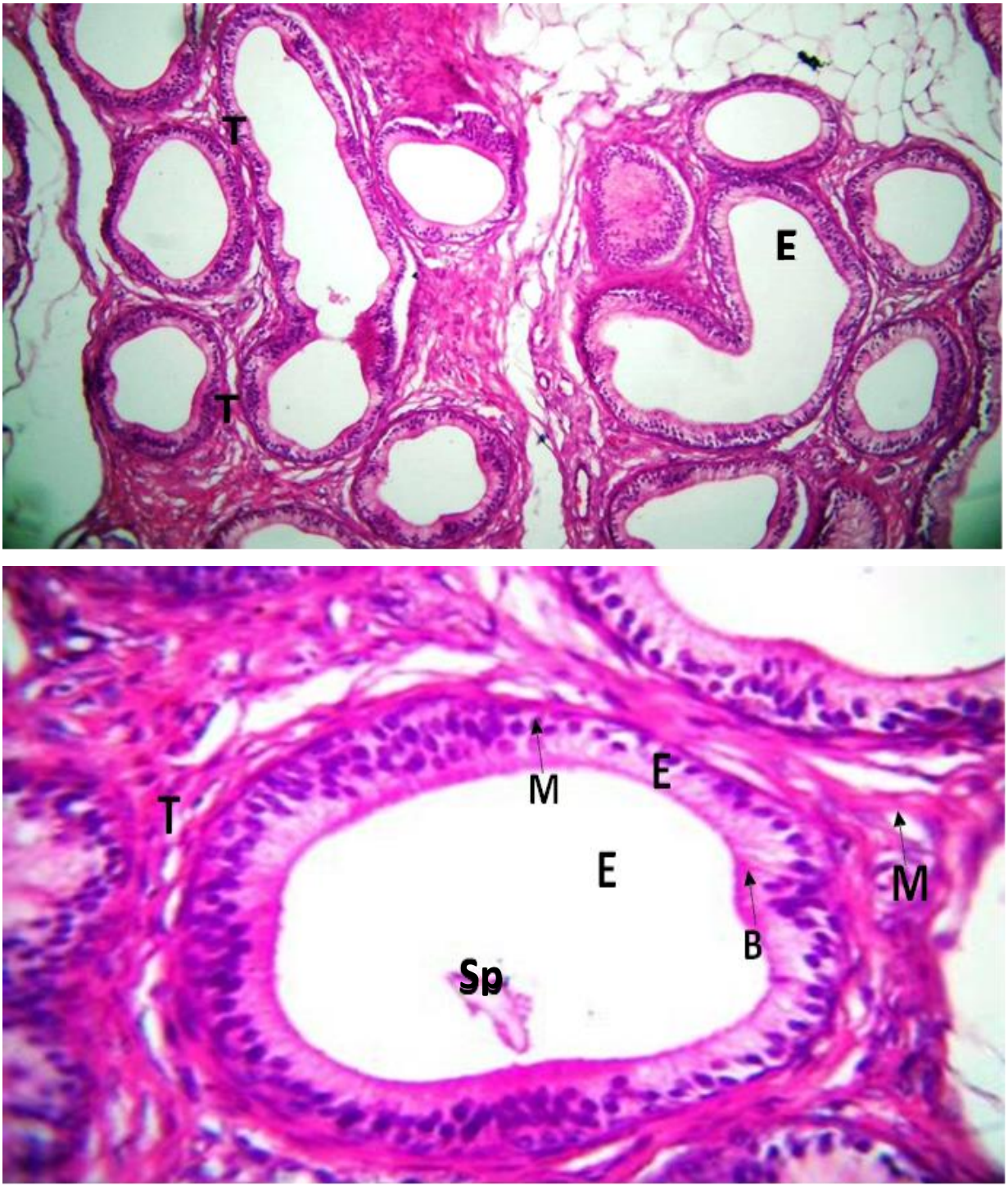


Plate 1: Histology of the epididymis in the control rabbit. Magnification (a: Gr x100 and b: Gr x400) **HE stained (TE):** Epididymal tubes**, (Spz):** Spermatozoa, **(Ep):** Epithelium epididymal, **(EL):** Epididymal light, **(TI)**: Interstitial tissue, **(MS)**: Smooth muscle, **(MC)**: Main cells, **(BC)**: Basal cells

Histology of epididymis in rabbits treated by ascorbic acid (HE coloring)

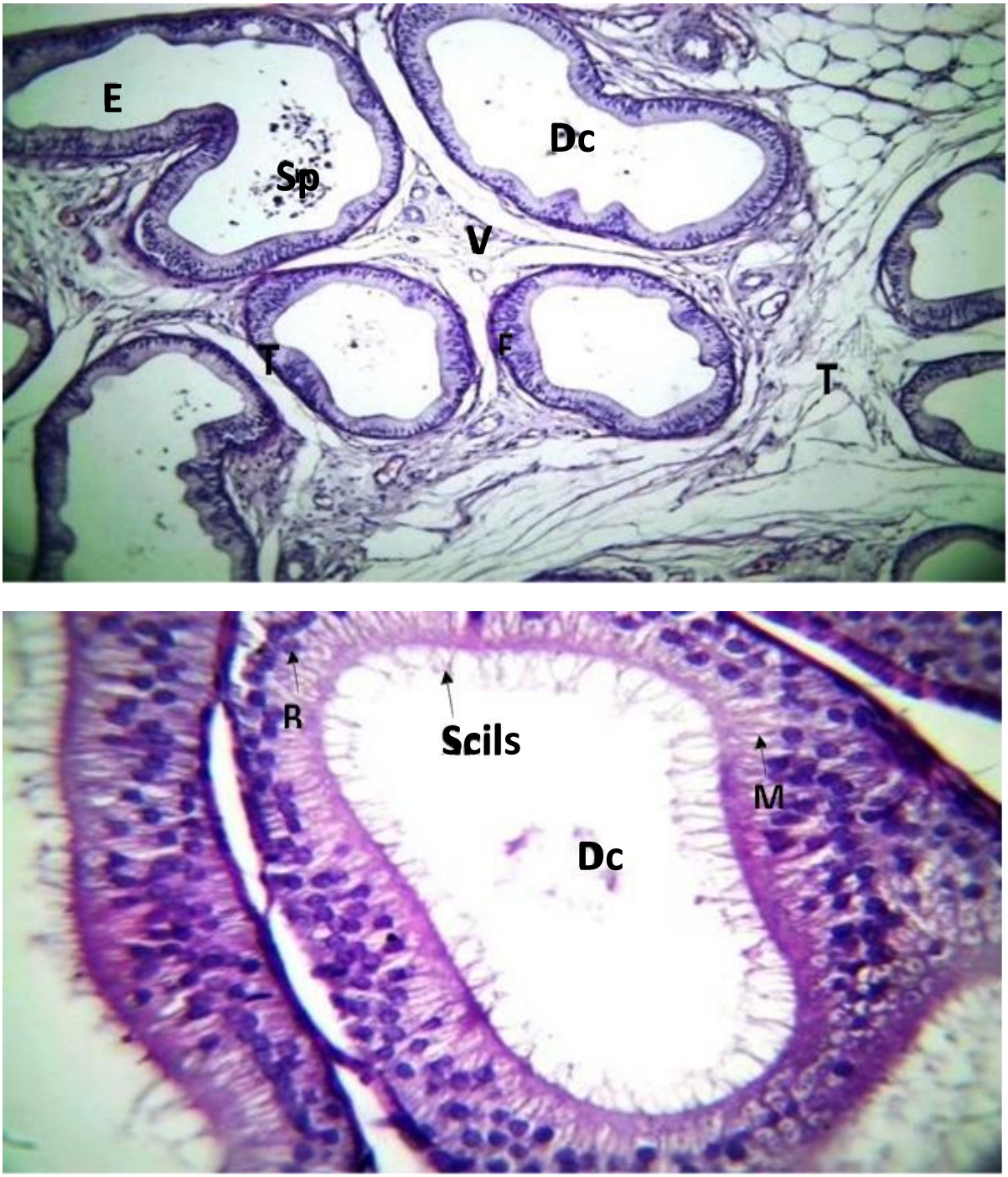


Plate 2: Histology of epididymal in rabbits treated with ascorbic acid. Magnification (a: Gr x100 and b: Gr x400). **HE stained**.

(TE): Epididymal tubes, **(Spz)**: Spermatozoa, **(Ep)**: Epididymal epithelium, **(EL)**: Epididymal light, **(TI)**: Interstitial tissues, **(BC)**: Basal cell, **(MC)**: Main cell, **(Scils)**: Stereocilia **(V)**: Vacuoles, **(Dc)**: Cell debris

Histology of epididymis in controls rabbits' colors by Masson trichrome coloring

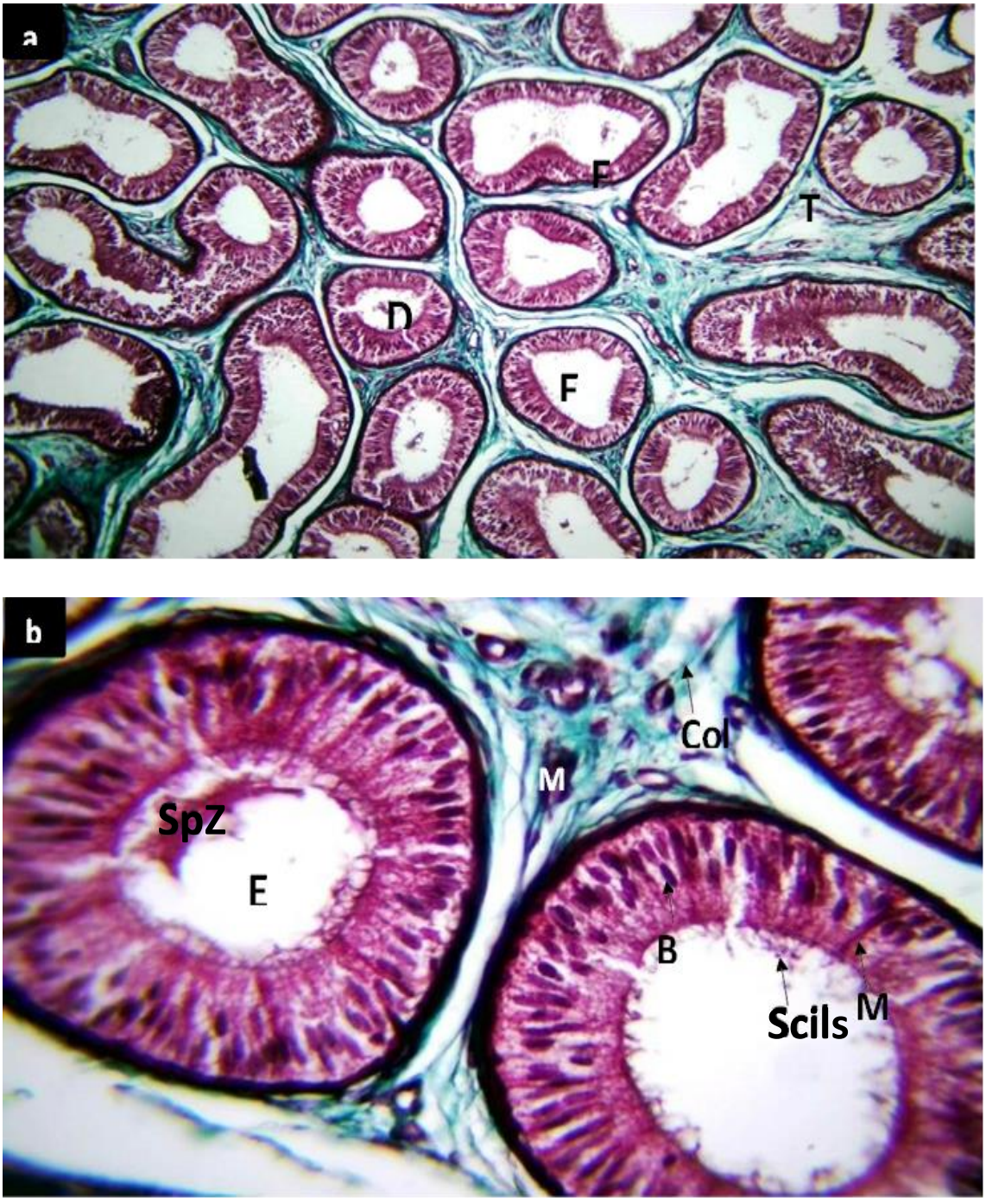
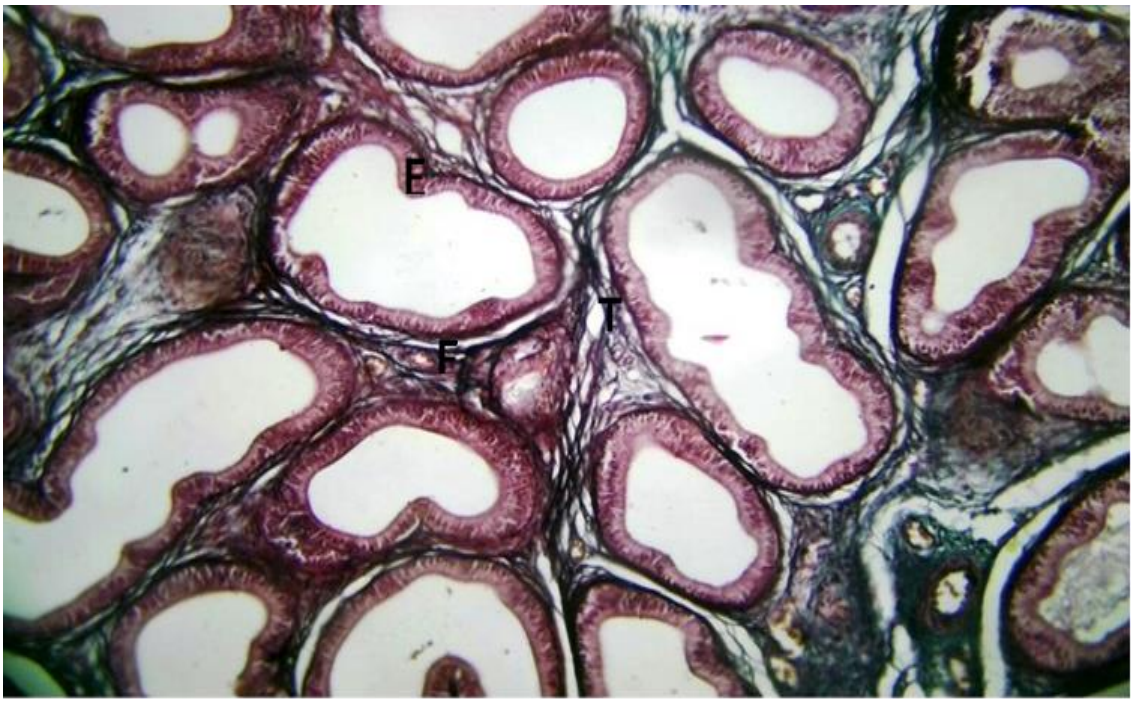


Plate 3: Histology of epididymal tissue of rabbits treated. Magnification (a: Gr x100 and b: Gr x400).by **Masson trichrome**.
(TE): Epididymal tubes, **(Ep)**: Epididymal epithelium, **(EL)**: Epididymal light, **(TI)**: Interstitial tissues, **(BC)**: Basal cell, **(MC)**: Main cell, **(Spz)**: Spermatozoa, **(Scils)**: Stereocilia, **(coll)**: collagen fibers, **(MS)**: Sooth muscle

Histology of epididymis in rabbits treated by ascorbic acid and colors by Masson trichrome coloring



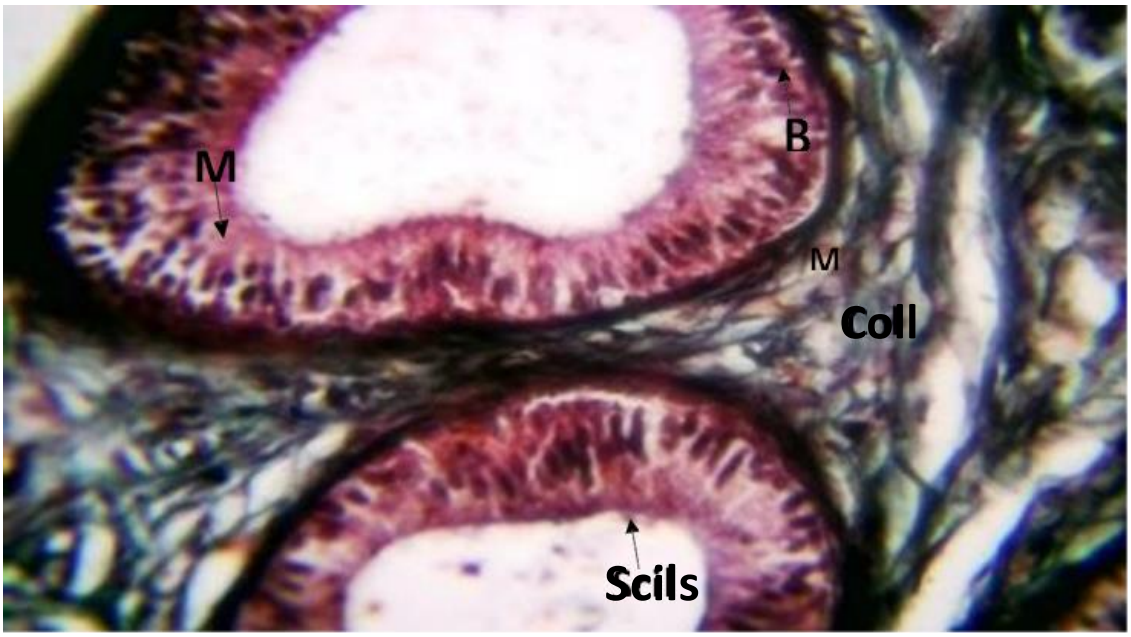


Plate 4: Histology of epididymal tissue of rabbits treated by Acid ascorbic stained with Masson trichrome. Magnification (a: Gr x100 and b: Gr x400).
(Ep): Epididymal epithelium, **(EL)**: Epididymal light, **(TI)**: Interstitial tissues, ,**(BC)**: basal cell, **(MC)**: Main cell, **(Scils)**: Stereocilia, **(coll)**: collagen fibers, **(MS)**: Smooth muscle

3. Immunohistochemical study (IHC)

3.1. IHC alpha-fetoprotein of epididymis of control rabbits

As mentioned in Plate 5, the microscopic observation of the sections low magnification (Gx400) and high magnification epididymis immunohistochemicals (Gx400) in groups of rabbits, following AFP immunostaining, the appearance of a blue coloration indicates the absence of AFP molecular expression in the epididymal epithelium. Therefore, the test result is negative. Also the appearance of a brown coloration Cell debris in the epididymal light level.

3.2. IHC alpha-fetoprotein of epididymis of rabbits treated with ascorbic acid

As mentioned in Plate 6 , the microscopic observation of the sections low magnification (Gx400) and high magnification epididymis immunohistochemicals (Gx400) in groups of rabbits , following AFP immunostaining treated by Ascorbic acid , An increase in diameter of the intertubular space and light of the tubes epididymal the appearance of a blue coloration indicates the absence of AFP molecular expression in the epididymal epithelium and The appearance of spermatozoa clusters(Spz) in the lumen of the epididymal tube(TE) with a blue coloration Therefore, the test result is negative.

IHC alpha-fetoprotein of epididymis of control rabbits

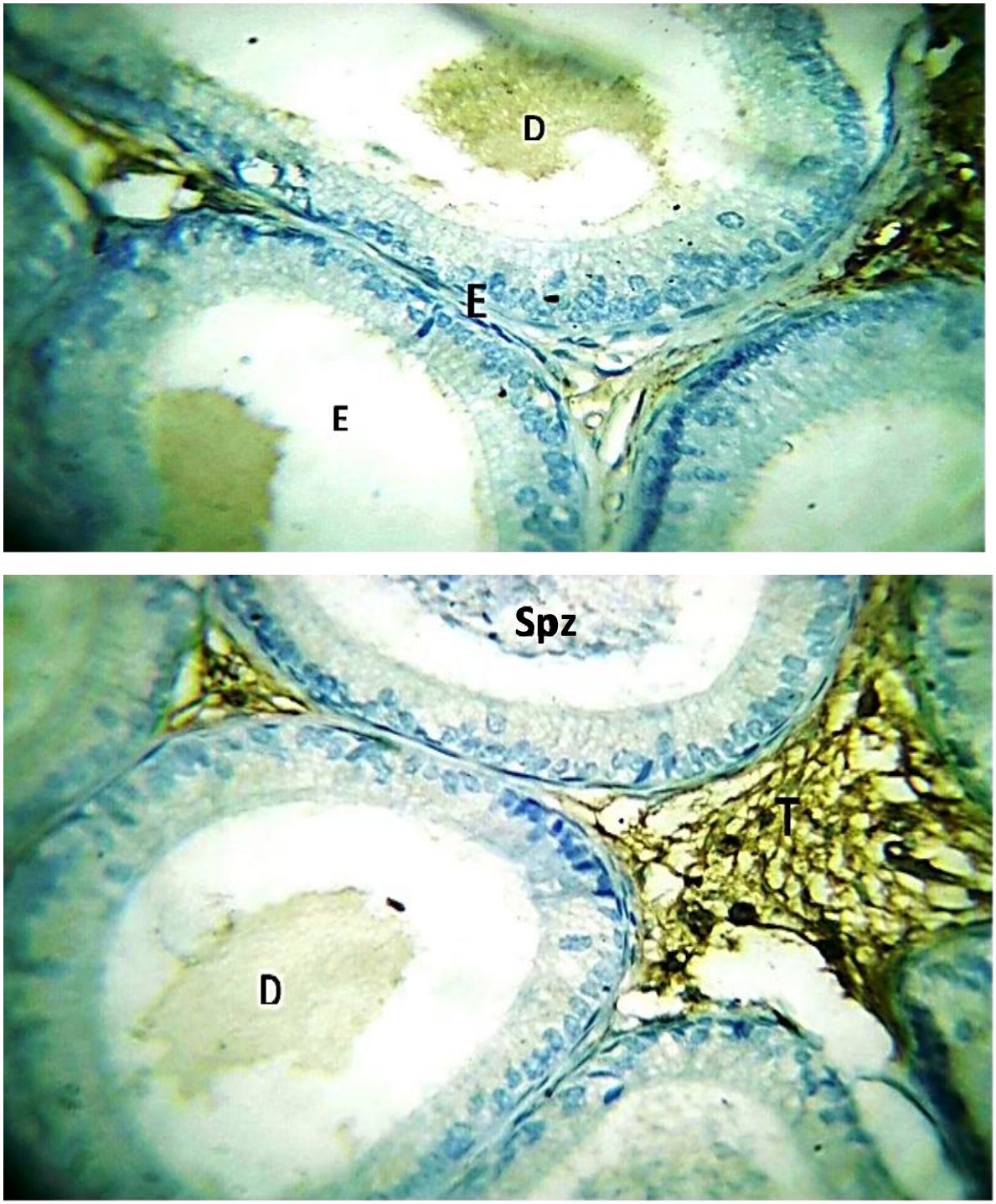


Plate 5: The microscopic observation of the sections low magnification (Gx400) and high magnification epididymis immunohistochemicals (Gx400) in groups of rabbits, following AFP immunostaining,
(EP): Epididymal epithelium, **(EL)**: Epididymal light, **(TI)**: Interstitial tissues, **(Spz)**: Spermatozoa, **(Dc)**: Cell debris

IHC alpha-fetoprotein of epididymis of rabbits treated with ascorbic acid

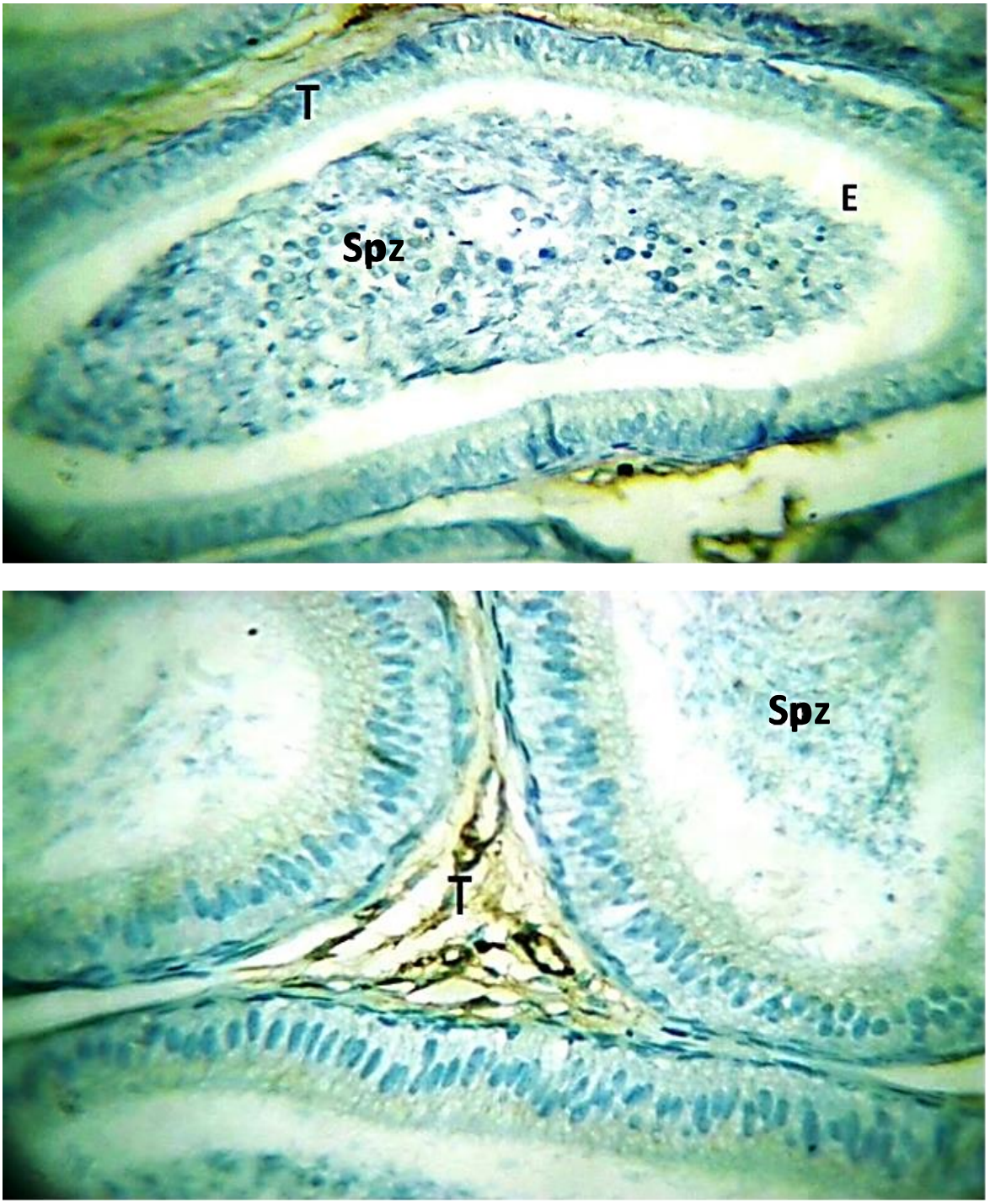


Plate 6: microscopic observation of the sections low magnification (Gx400) and high magnification epididymis immunohistochemicals (Gx400) in groups of rabbits, following AFP immunostaining treated by Ascorbic acid.
(TE): Epididymis tubes, **(EL):** Epididymis light, **(TI)**: Interstitial tissues, **(Spz)**: Spermatozoa.

4. Molecular docking Results

Our approach consists of a molecular docking of ascorbic acid (vitamin C) and AFP (alpha-fetoprotein). In this study, we calculated their binding energies to assess the affinity between these two molecules.

When the docking link **energy** is **lower,** the binding **affinity** for this target is **highest.**

4.1. molecular docking analysis

In this study, we used AutoDock Vina to estimate the binding affinity between ligand ascorbic acid, and protein alpha-fetoprotein (AFP). AutoDock Vina can predict interactions and binding energies based on different docking configurations. The binding affinities obtained for the most favorable mode are presented in Table 1. This table summarizes the values of ligand binding energies calculated by AutoDock Vina, providing crucial information on the stability and potential effectiveness of this molecular interaction. These results may have important implications for the design of drugs or diagnostic tools based on the interaction between ascorbic acid and AFP.

- **Affinity (kcal/mol)**: This figure represents free binding energy, or vitamin C affinity for alpha-fetoprotein. The more negative this number, the more favorable and stable the interaction. Here, the affinity varies from -7.5 to -6.4 kcal/mol.
- **Dist from best mode (RMSD l.b. and u.b.)**: RMSD stands for "Root Mean Square Deviation" and measures the difference between the conformations of the resulting poses. RMSD l.b. (lower bound) and u.b. (upper bound) indicate the accuracy of the pose compared to the best pose found (the first).

Table VII: Affinity of ascorbic acid with 7YIM receptor.

Mode	Affinity (Kcal/mol)	Distance from best mode	
		rmsd l.b.	rmsd u.b.
1	-7.5	0.000	0.000
2	-7.3	38.421	38.850
3	-7.2	25.178	25.949
4	-7.1	1.625	2.193
5	-6.9	53.651	54.471
6	-6.8	26.432	27.238

7	-6.6	25.894	26.661
8	-6.5	53.066	53.906
9	-6.4	26.794	27.502

They are shown to:

- **Mode 1:** This pose has the highest energy affinity (-7.5 kcal/mol) and therefore the most stable bond. The RMSD values (0.000) indicate that it is considered the reference for other exposures.
- **Mode 2:** Affinity of -7.3 kcal/mol with high RMSD (38.421, 38.850). This means that this pose is much less similar to pose 1 in terms of spatial conformation.
- **Mode 3:** Affinity of -7.2 kcal/mol with moderate RMSD (25.178, 25.949). This is a relatively stable pose but different from pose 1.
- **Mode 4:** Affinity of -7.1 kcal/mol with low RMSD (1.625, 2.193). This pose is very close to pose 1 in terms of conformation.
- **Mode 5:** Affinity of -6.9 kcal/mol with very high RMSD (53.651, 54.471). This pose is very different from pose 1.
- **Mode 6:** Affinity of -6.8 kcal/mol with moderate RMSD (26.432, 27.238).
- **Mode 7:** Affinity of -6.6 kcal/mol with moderate RMSD (25.894, 26.661).
- **Mode 8:** Affinity of -6.5 kcal/mol with very high RMSD (53.066, 53.906).
- **Mode 9:** Affinity of -6.4 kcal/mol with moderate RMSD (26.794, 27.502).

✓ **General Interpretation:**

- Mode 1 is the best mode in terms of binding energy, indicating the most stable conformation.
- Mode with low RMSD compared to mode 1 (such as mode 4) may represent similar and potentially biologically relevant alternative conformations.
- Mode with very high RMSD (such as mode 2, 5, and 8) are significantly different and may represent less likely conformations.

4.2.Protein-ligand interactions (AFP interaction and ascorbic acid)

Molecular docking studies were performed to thoroughly analyze the interactions between ascorbic acid and the AFP protein (Alpha-FoetoProtéine).

In this step, we analyzed the interactions between ligand, ascorbic acid, and AFP protein using Discovery Studio software. This analysis allowed us to understand how the ligand is positioned in the active site of t.he protein and to identify the residues of the active site involved in protein-ligand interactions. We also determined the types of bonds formed, including hydrogen and hydrophobic bonds.

We determine two best modes (the least distance) of interactions: mode 1 and 4.

- **Mode 1**

Table VIII: The characteristics of AFP-vit C complex and the number of hydrogen and hydrophobic bonds formed in protein-ligand interactions in mode1.

	Name	Distance	Categories	types
1.	ASP276	2,63205	Hydrogen/hydrophobic bond	Salt Bridge;Attractive Charge
2.	ASP280	2,60242	Hydrogen/hydrophobic bond	Salt Bridge;Attractive Charge
3.	ASP276	4,82842	hydrophobic	Attractive Charge
4.	ASP276	2,86627	hydrophobic	Attractive Charge
5.	ASP276	3,04219	hydrophobic	Attractive Charge
6.	ASP280	3,96631	hydrophobic	Attractive Charge
7.	ASP280	2,98944	hydrophobic	Attractive Charge
8.	ASP280	2,88458	hydrophobic	Attractive Charge
9.	ASP33	4,75983	hydrophobic	Attractive Charge
10.	CYS86	3,52006	Hydrogen Bond	Conventional Hydrogen Bond
11.	LYS283	2,5852	Hydrogen Bond	Conventional Hydrogen Bond

Hydrogen bonds are crucial for protein-ligand interactions.

Vitamin C obtained the greatest interaction by forming 04 hydrogen bonds with the residues ASP276, ASP280, CYS86 and LYS283 of the AFP receptor.

As shown in Figure , vitamin C forms 04 hydrophobic bonds with the ASP276 residue at a distance of 2.63Å, 4.82, 2.86 and 3.04Å, and 04 other hydrophobic bonds with the ASP280 residue of the 2.60Å remote AFP receptor, 3.96, 2.98 and 2.88Å, and a hydrophobic bond with the ASP33 residue at distance 4.75 Å

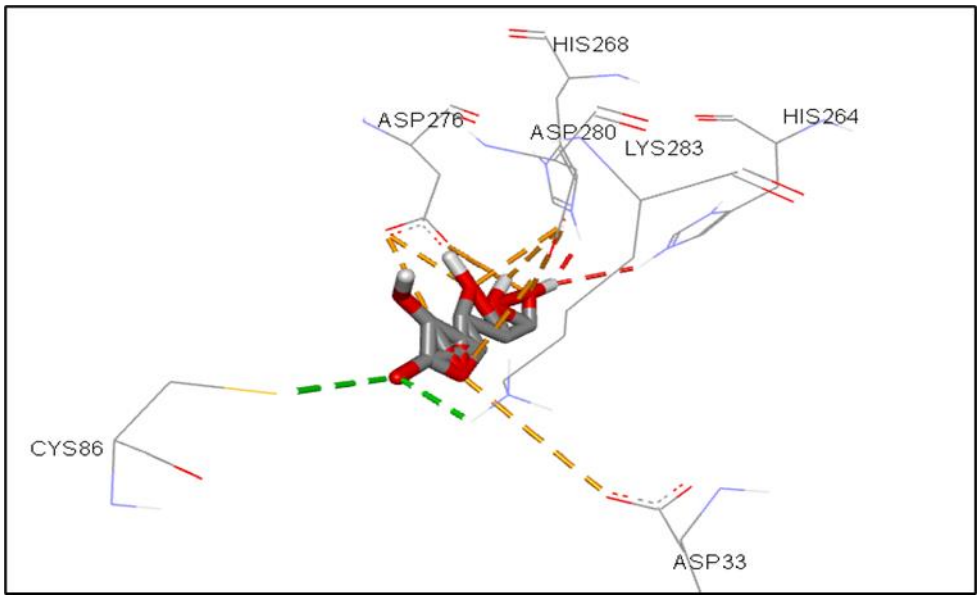


Figure 10: 3D structure of ascorbic acid with AFP receptor in mode 1

- **Mode 4**

Table IX: The characteristics of AFP-vit C complex and the number of hydrogen and hydrophobic bonds formed in protein-ligand interactionsin mode 4.

	Name	**Distance**	**Categories**	**types**
1.	ASP280	2,46782	Hydrogen / hydrophobic Bond	Salt Bridge;Attractive Charge
2.	ASP33	5,48966	hydrophobic	Attractive Charge
3.	ASP276	3,83106	hydrophobic	Attractive Charge
4.	ASP280	4,67113	hydrophobic	Attractive Charge
5.	ASP33	4,40066	hydrophobic	Attractive Charge
6.	ASP276	5,12322	hydrophobic	Attractive Charge
7.	ASP33	4,90999	hydrophobic	Attractive Charge
8.	ASP276	4,71262	hydrophobic	Attractive Charge
9.	ASP280	2,74634	hydrophobic	Attractive Charge
10.	ASP33	3,65334	hydrophobic	Attractive Charge
11.	ASP280	2,86024	hydrophobic	Attractive Charge
12.	ARG19	3,03849	Hydrogen Bond	Conventional Hydrogen Bond
13.	ASP276	3,3185	Hydrogen Bond	Conventional Hydrogen Bond
14.	ASP276	3,23732	Hydrogen Bond	Conventional Hydrogen Bond
15.	LEU32	2,28	Hydrogen Bond	Conventional Hydrogen Bond

Vitamin C obtained the greatest interaction by forming 05hydrogen bonds with the residues ASP280, ARG19, ASP276 and LEU32 of the AFP receptor.

As shown in Figure , vitamin C forms 4 hydrophobic bonds with the ASP280 residue at a distance of 2.46Å, 4.67 , 2.74 and 2.86Å, and 4 hydrophobic bonds with the ASP33 residue of the 5.48Å remote AFP receptor,4.40 ,4.90 and 3.65Å, and 3 hydrophobic bonds with the ASP276 residue at a distance of 3.83 Å ,5.12 and 4.71 Å.

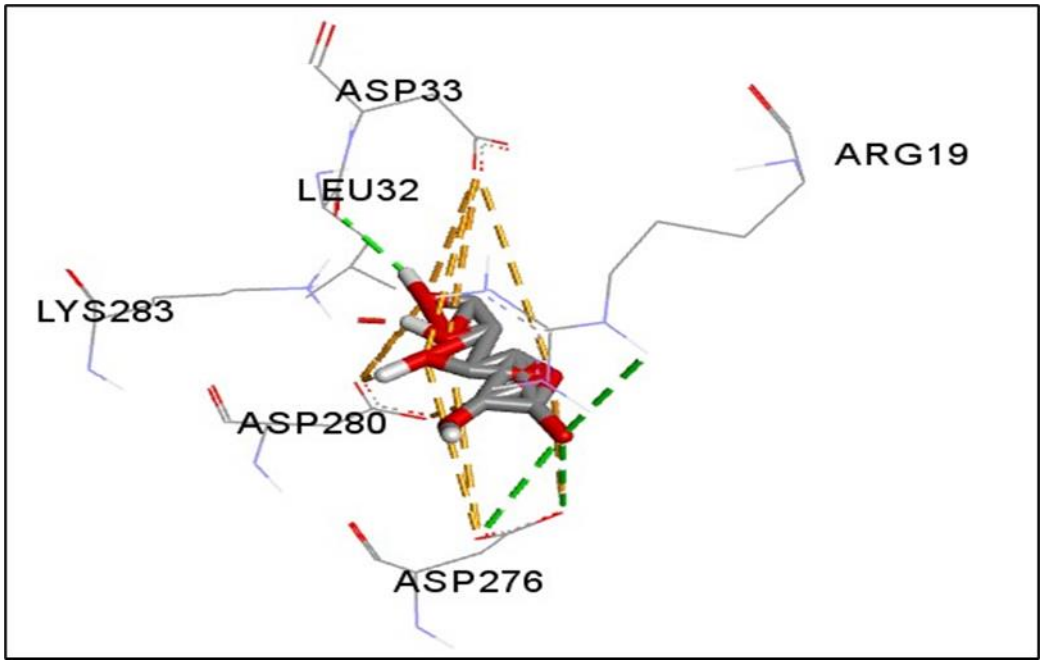


Figure 11: 3D structure of ascorbic acid with AFP receptor in mode 2

5. BIOINFORMATICS RESULTS

Bioinformatics analysis of alpha-fetoprotein in the rabbit

Very little data concerning alpha-fetoprotein are deposited on the various databases data on the different model organisms in the know the rabbit.

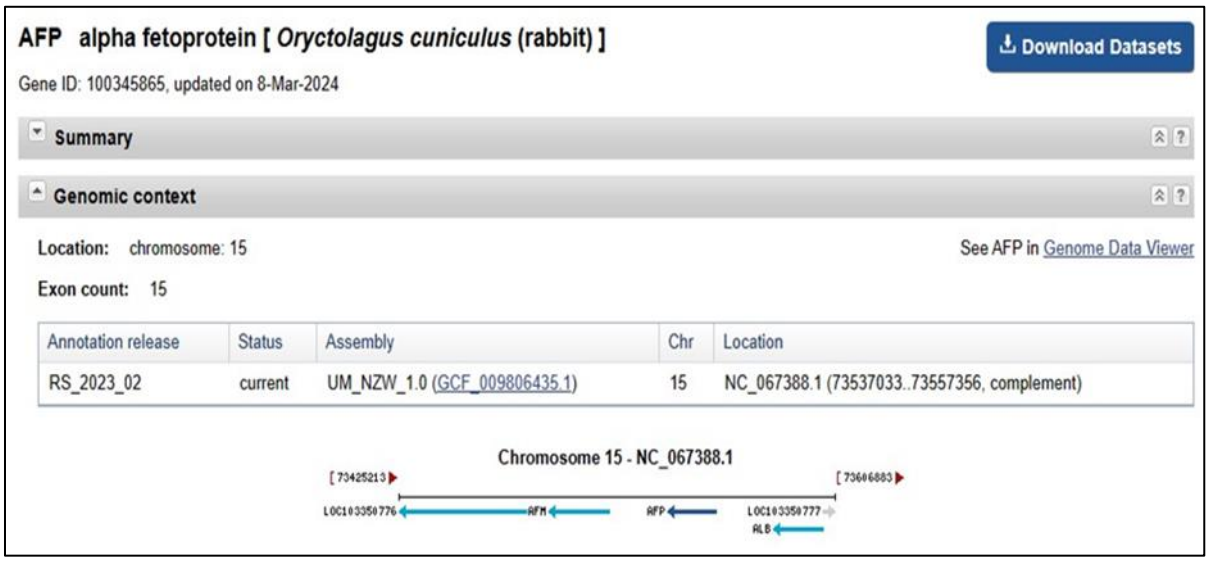


Figure 12: Location gene AFP in rabbit

FASTA sequence and 3D AFP structure

The AFP FASTA sequence is as follows:

```
>XP_002717072.1 alpha-fetoprotein [Oryctolagus cuniculus]
MKWVVSIFLLLLNFTESKTLHSNAYGIDSILDSSKCSAGMNLVDLATIFVTQFAQEATYEEISKMVRDVL
TIIEKPTVRDQSSGCLENQLPAFLEEICHETEISEKCGLAECCRQGGDDRHRCFLAHKKAAHASIPPFQV
PEPVSGCKAYEENREAFMNRYIYEISRRHPFLYAPTILSLATRYDKIIPPCCKAENAVECFQTKAAPITK
ELREVSLLNQHVCSVMRNFSPLTFQAITVVKMSQKLPKANFTEIQKLVLDVAHTHKECCQGNVLECLQDG
ERIMSYICSQQHILPSTIAECCKLPILELGHCIIHAENDDKPEGLSPHLHRFLGDRDFNQFSSQQKNIFL
ASFVYEYSRRHPELPVTVILRITKGYQELLEKCFQTGNPLECQDKGEEELQKYIQESQAVVKRSCDLYQK
LGEYNFQNVFLVSYTKKAPQLTSPELIALTRKMAAKAAMCCHLSEDKRLACAETAADLVLGQLCIRHEAT
PVNPGVGQCCTSSYANRALCFNKLVLDETYVPPPFSADKFIFHADLCQAQGIALQTMKQEFLINLVKQKP
QITQEQLEAVIADFSGMLEKCCHGQEREACFAQEGPKLISKTRAALEV
```

The 3d structure of the AFP obtained from Swiss Model is shown in the figure below

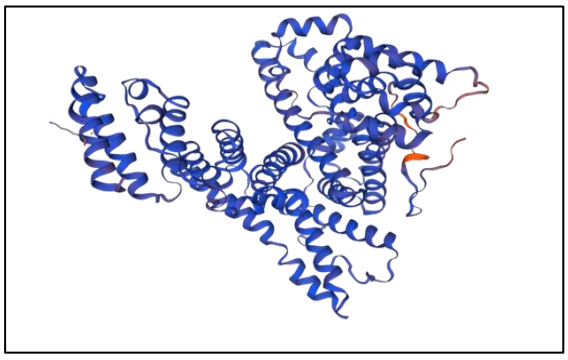
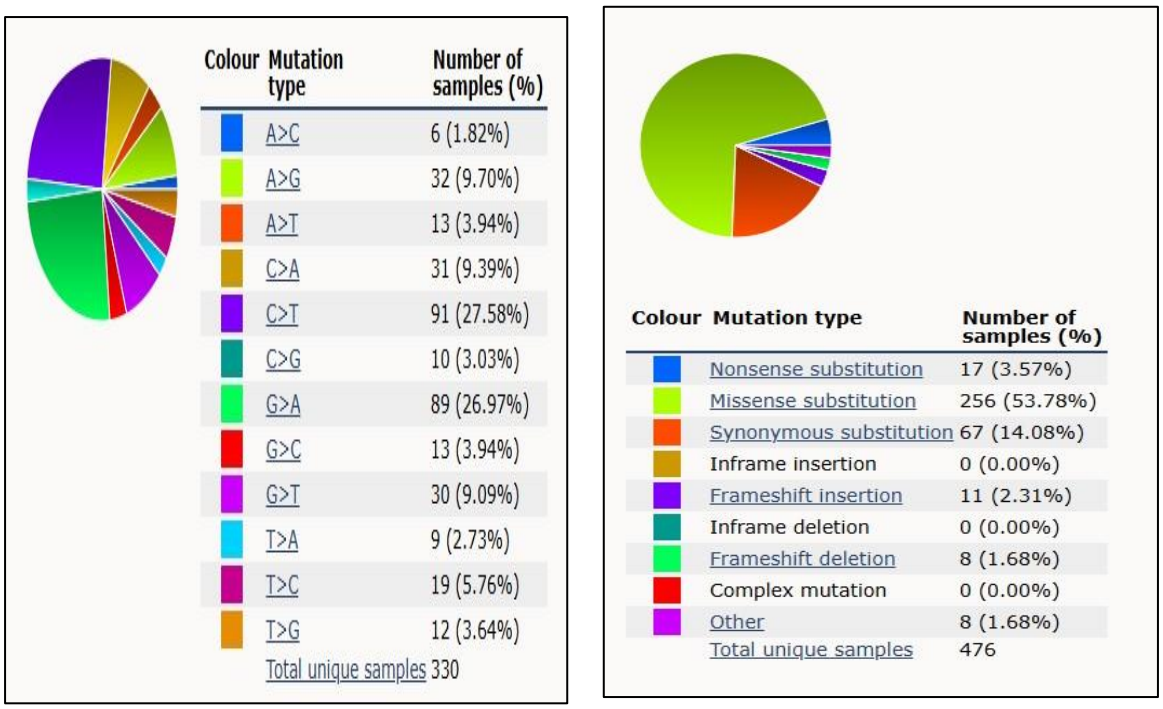


Figure 13: 3D AFP structure



Colour	Mutation type	Number of samples (%)
	A>C	6 (1.82%)
	A>G	32 (9.70%)
	A>T	13 (3.94%)
	C>A	31 (9.39%)
	C>T	91 (27.58%)
	C>G	10 (3.03%)
	G>A	89 (26.97%)
	G>C	13 (3.94%)
	G>T	30 (9.09%)
	T>A	9 (2.73%)
	T>C	19 (5.76%)
	T>G	12 (3.64%)
	Total unique samples	330

Colour	Mutation type	Number of samples (%)
	Nonsense substitution	17 (3.57%)
	Missense substitution	256 (53.78%)
	Synonymous substitution	67 (14.08%)
	Inframe insertion	0 (0.00%)
	Frameshift insertion	11 (2.31%)
	Inframe deletion	0 (0.00%)
	Frameshift deletion	8 (1.68%)
	Complex mutation	0 (0.00%)
	Other	8 (1.68%)
	Total unique samples	476

Figure 14: Types of mutations on gene AFP in human

Discussion

The present study focuses on the evaluation of histological and immune-histochemical alterations of the epididymis in the distal region of the head in the male rabbit of local strain "*Oryctolagus cunniculus*" following vitamin C supplementation. Specifically, we investigated the effect of ascorbic acid, known for its antioxidant properties, on alpha-fetoprotein (AFP) expression. We used an immune-histochemical approach to evaluate the expression of AFP a protein involved in various biological processes carcinogenesis in tissues Its expression can be influenced by various factors, including environmental factors such as antioxidants like vitamin C, We also performed a bioinformatics study of AFP expression to reinforce our observations. By integrating experimental observations with bioinformatics analysis, we expect to elucidate the effects of vitamin C supplementation on various aspects of epididymis biology. This comprehensive approach will allow us to explore molecular interactions, signaling pathways and potential functional implications in a more holistic manner. Through this combined effort, we aim to gain insight into how vitamin C influences epididymis physiology, including its role in sperm maturation, storage and other related processes.

The epididymis is involved in the transport and maturation of sperm and, in its terminal part (tail or cauda), in the storage of sperm between two ejaculations

Motility and fertilizing power of sperm increase for sperm collected in the more distal regions of the epididymis tubule. Associated with this post-testicular functional maturation of spermatozoa, the epididymis also plays a role of «quality control» **(Noblanc et *al*., 2012).**

Histological examination of epididymis in rabbits treated with ascorbic acid for 21 days and stained with HE coloration revealed several alterations: The epididymis tubes (ET) have an irregular contour. The appearance of the Stereocilia (Scils). The appearance of the vacuols around the cells. The presence of accumulation of cell debris (Dc) at the epididymis light level of the tubes. An increase in diameter of the intertubular space and light of the tubes epididymis. The appearance of sperm clusters (Spz). However, no study to date has explored the effects of ascorbic acid exposure on epididymis histology in rabbits

Histological examination of epididymis in rabbits treated with ascorbic acid for 21 days and stained with trichromatic masson coloration revealed several alterations: present a regularly contoured epididymis tubule (ET) , an increase in tubular lumen diameter , also the degeneration of sperm clusters in the epididymis epithelium , the light level becomes clear

with degeneration of accumulation of cell debris (Dc) in the epididymis light level , also decreases in epididymis epithelium leads to an increase in the light level , the scils have almost disappeared , the appearance of collagen fibres with green color.

The immune-histochemical study of the epididymis in rabbits treated with ascorbic acid reveals a negative cytoplasmic staining with the anti-AFP antibody in marked retracted tissues.

Ascorbic acid is an electron donor and acts as a cofactor for fifteen mammalian enzymes **(Padayatty et Levine, 2016).**

Ascorbic acid or Vitamin C is very important in our body because of its antioxidant property also known to play countless functions for maintaining optimal health to protect the human from various diseases **(Gupta et *al*., 2022).**

The protective role of vitamin C in cancer progression has historically been attributed to its antioxidant activity and the prevention of DNA damage induced by oxidative stress **(Brabson et *al*., 2021).**

Vitamin C is a superior scavenger of AEO (HO• or O2 •), and it also prevents lipid peroxidation by producing vitamin E from the radical form that is created when it interacts with lipid radicals. Its functions are numerous: contributing to the proper functioning of the immune system, involvement in the synthesis of collagen and red blood cells, as well as in the mechanisms of iron metabolism **(Kaźmierczak-Barańska et *al*., 2020).**

Vitamin C supplements are beneficial in many ways that can affect fertility of men, such as reducing ROS concentration, increasing sperm quality, decreasing sperm DNA oxidation, and decreasing sperm membrane lipid peroxidation. The quality of sperm can be enhanced by taking Vitamin C supplements as antioxidants in a dose-dependent **(Gupta et *al*., 2022).**

The creation of oxidative stress is a result of an imbalance between the body's antioxidant defense capabilities and the production of reactive oxygen species (ROS). To protect themselves from oxidative stress, organisms have developed an arsenal of antioxidants, including enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and vitamins such as A, E, and C **(Baudin, 2020).**

Oxidative stress has become the focus of interest as a potential cause of male infertility. Normally, equilibrium exists between reactive oxygen species (ROS) production and antioxidant scavenging activities in the male reproductive organs **(Vijayprasad et *al*., 2014).**

The ascorbic acid is a known antioxidant present in the testis with the precise role of protecting the latter from the oxidative damage. It also contributes to the support of spermatogenesis at least in part through its capacity to maintain this antioxidant in an active state **(Vijayprasad et *al*., 2014).**

Alpha-foetoprotein (AFP), one of the first protein tumour markers discovered **(Sauzay et *al*., 2016).**

AFP is an oncofetal glycoprotein belonging to the superfamily of albuminoids, which also includes albumin, the Gc globulin protein (vitamin D binding protein), AFP is a molecule involved in proliferation, angiogenesis and apoptosis **(Mitsuhashi et *al*., 2008) (Desbene et Gaillard 2013).**

AFP immune-reactivity has been detected in the cytoplasm of tumor cells. These features have been noted in many reports of human and rodent testicular yolk sac carcinoma. Therefore, AFP immune-histochemical staining would be very useful in differentiating testicular yolk sac carcinoma in domestic animals **(Kagawa et *al.*, 1998).**

Conclusion

The work conducted enabled us to assess to the impact of ascorbic acid supplementation on the epididymis tissue of rabbits (*Oryctolagus cuniculus*), Vitamin C known for its antioxidant properties, plays a crucial role in protecting cells from oxidative stress, including those in the epididymis crucial for sperm maturation and storage.

The findings from studies on rabbit epididymis histology after vitamin C supplementation highlight significant alterations in tissue structure, including irregularities in epididymis tubules, changes in tubular lumen diameter, and signs of sperm cluster degeneration. While these observations suggest potential negative impacts on epididymis morphology, they also underscore vitamin C's complex role in male reproductive health.

Additionally, vitamin C may be involved in regulating various biochemical and metabolic processes critical for sperm quality and overall male fertility.

These findings highlight the complex interplay between antioxidant supplementation and male reproductive health, prompting the need for deeper molecular and long-term studies to elucidate the precise mechanisms and clinical implications. Understanding these histological changes is crucial for optimizing ascorbic acid's therapeutic potential in managing epididymis-related disorders and enhancing male fertility outcomes.

The results of an immune-histochemical study that examined the expression of alpha-fetoprotein (AFP) in the epididymis of male rabbits after supplementing with vitamin C were negative.

Molecular docking studies were conducted to thoroughly analyze the interactions between ascorbic acid and the AFP protein (Alpha-Fetoprotein), this analysis allowed us to understand how the ligand is positioned in the active site of the protein and to identify the residues of the active site involved in protein-ligand interactions. We also determined the types of bonds formed, including hydrogen and hydrophobic bonds.

In perspective, it would be interesting to:

- Explore further how vitamin C specifically influences epididymis histology and sperm function at a molecular level.

- Understanding the genetic location of AFP in rabbits sheds light on its evolutionary and functional role in non-human mammals.
- Mutation analysis helps identify mechanisms of diseases such as cancer and find potential therapeutic targets.
- Determine the most effective treatment approaches for reproductive health by comparing vitamin C to other antioxidants.

A

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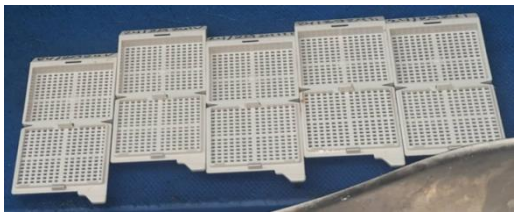
ANNAXES

The cassettes	
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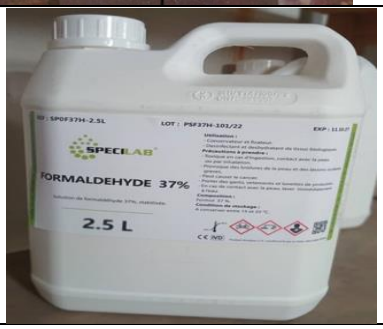
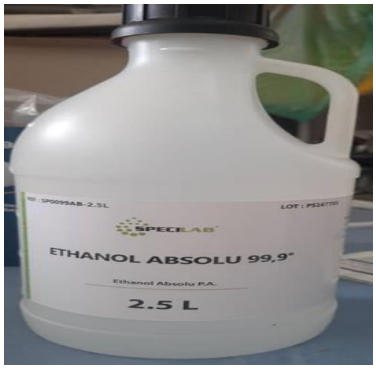
Formol	
Ethanol	

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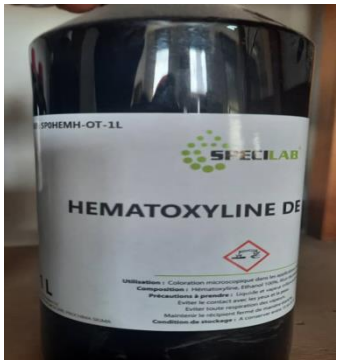
Haematoxylin	
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