الجمهورية الجزائرية الديمقراطية الشعبية

DEMOCRATIC AND POPULAR ALGERIAN REPUBLIC

Serial N°:

وزارة التعليم العالي والبحث العلمي

MINISTRY OF HIGHER EDUCATION AND SCIENTIFIC RESEARCH

جامعة الشهيد حمه لخضر الوادي

ECHAHID HAMMA LAKHDAR UNIVERSITY OF EL-OUED

كلية العلوم الطبيعية والحياة

FACULTY OF NATURAL SCIENCES AND LIFE

قسم البيولوجيا الخلوية والجزيئية

DEPARTMENT OF CELLULAR AND MOLECULAR BIOLOGY

Master's Thesis

In order to obtain a diploma of an Academic Master In biological sciences

Specialty: Applied Biochemistry

Theme

Study of the effects of a Novel ZnONPs synthesized by biological and chemical methods compared to zinc salts on Cyclophosphamide-induced oxidative stress , immunosuppression and biochemical disorder in Wistar rats

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University year: 2021/ 2022


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إهْـــــــــــــــــــــــــــدَاء

الحمد لله الحمد لله الحمد لله

الحمد لله "كريم الفضل ، عظيم الاحسان" على سعة النعم و كثرة العطايا ، الحمد لله الذي منّ علينا بإتمام هذا العمل و لولاه لما جرى القلم ...

أما بعد : إنه لشرف عظيم أن أتقدّم بإهداء هذا العمل العلمي المتواضع الى :

مهجة القلب و الروح و بلسم الجروح ، إلى الملاذ و الأمان و السلام

إلى مطلع المعجزات و حجّة الله في الكائنات

إلى تربةٍ تاه فيها الجلال فتاهت بها القمم الشامخات

إلى الغالي وطني الحبيب " الجزائر " الروح و الدم فداه

إلى الذين رسموا حدود هذا الوطن و إلى دمائهم الزّاكيات الطّاهرات

إلى ألّذين علمونا الكتابة بالّدم

إلى الّذين غادروا عالمنا و لم يغادرونا

إلى شهدائنا الأبرار : العربي بن المهيدي ، مصطفى بن بولعيد ، زيغود يوسف ، ديدوش مراد ،حسيبة بن بوعلي رابح بيطاط . و و الله لو كان لي من المكان متسع لذكرتكم شهيدًا تلو الآخر ...

إلى كل من ذرف دمعًا و نزف دمًا لأجل هذا الوطن...

إلى وطني الصّغير : عائلـــــــــــــــــــــــــــــتي

Acknowledgment

الشكر لله أولا و دائما و ابدا الحمد لله الذي لا حول لنا و لا قوة إلا به عز و جل

Then , I express my highest thanks and gratitude to our teacher who taught us the most interesting and biochemical lessons : Dr. DEROUICHE Samir for his great bounty and for this - very - important and great research …

We would like to thank our thesis committee: Dr. LAICHE Omar Touhami and Dr. ZEGHIB Khaoula for honoring them as members of the discussion committee and enriching our work with their evaluation and constructive comments.

I also express my thanks to the workers of the Cancer Control Center in general and to I.Amer in particular . As well , Laboratory officials of the faculty of Natural and Life Sciences , And the member of the research laboratory at the faculty of Science and Technology : Mr.TLIBA Ali . Also to to the doctors : MAALOULE Toufik and FARDIYA .

Thank you to everyone who supported us with their expertise and experience, on top of them: Dr. Djouadi Anfal , Dr. LAIB Ibtissam and Dr.CHOUIA Amel and to future teachers : C.Marwa and B.Wissem

And for those who supported us morally always : G.Nadjwa and H.Oumaima

The most beautiful

bouquets of roses for you...

Abstract

There are many substances that are used in chemotherapy anti-cancer, including cyclophosphamide, which is known to be highly effective, but with severe secondary effects. The aim of our study is to evaluate the therapeutic effect of zinc nanoparticles (synthesized with biological and chemical methods) and zinc salt on biochemical disturbances, immunosuppression and oxidative stress (in liver and testis) induced by cyclophosphamide in rats.

For this purpose, twenty-five male Wistar rats were divided, into five groups (5 rats in each); healthy rats (Control), untreated cyclophosphamide rats (CPA) (30 mg/kg b.w./day for 3 days), Cyclo rats treated with biological zinc nanoparticles (CPA+AS-ZnNPs) (7 mg/kg b.w./week), Cyclo rats treated with chemical zinc nanoparticles (CPA+K-ZnNPs) (7 mg/kg b.w./week) and Cyclo rats treated with zinc salt (CPA+Zn nitrate) (54mg/kg b.w). All types of treatments were given to rats by injection except zinc nitrate orally for three weeks. Various parameters as hematological, biochemical and oxidative stress markers were estimated. Histopathology of testis and liver tissues was observed. Some characterization parameters and in-vitro study of nanoparticles were analyzed using standard protocols.

Results of in-vitro study proved the formation of nanoparticles by chemical and biological methods, with important antioxidant and anti-inflammatory activities. Results of in-vivo study in rats, show that cyclophosphamide treated rats induced an alteration in immunity cells, hemoglobin, red blood cells, testosterone and biochemical parameters compared to control group. Also cyclophosphamide treated rat's induced oxidative stress and histological alteration in testis and liver cells compared to control rats. Treatment of cyclophosphamide rats with zinc gave varying results with a preference for zinc synthetized by biological method (ascorbic acid), where we noticed a significant improvement in most of the previous parameters. This study indicated that the anti-inflammatory and antioxidant property of a novel AS-ZnNPs allowed using them to protect liver and testis from the side effects of cyclophosphamide or other chemotherapy drugs.

Key words: Cyclophosphamide, AA-ZnONPs, Testis, Liver, oxidative stress.

الملخص

هناك العديد من المواد المستخدمة في العلاج الكيميائي المضاد للسرطان ، بما في ذلك السيكلوفوسفاميد ، المعروف بفعاليته العالية ، ولكن له تأثيرات ثانوية حادة . الهدف من دراستنا هو تقييم التأثير العلاجي لجزيئات الزنك النانوية (المُصنَّعة بالطرق البيولوجية والكيميائية) وملح الزنك على الاضطرابات الكيميائية الحيوية ، وتثبيط المناعة ، والإجهاد التأكسدي (في الكبد والخصية) الناجم عن السيكلوفوسفاميد في الفئران .

لهذا الغرض ، تم تقسيم خمسة وعشرين ذكور جرذان ويستار ، إلى خمس مجموعات (5 فئران في كل مجموعة) ؛ فئران صحية (مجموعة تحكم) ، فئران سيكلوفوسفاميد غير معالجة (30 مجم / كجم من وزن الجسم / يوم لمدة 3 أيام) ، فئران السيكلو المعالجة بجزيئات الزنك الحيوية (CPA + AS-ZnNPs) (7مجم / كجم من وزن الجسم / أسبوع) ، فئران السيكلو المعالجة بجزيئات الزنك الكيميائية النانوية (CPA + K-ZnNPs) (7مجم / كجم من وزن الجسم / الأسبوع) وفئران السيكلو المعالجة بملح الزنك (CPA + نترات الزنك) (54 مجم / كجم من وزن الجسم). أعطيت جميع أنواع العلاجات للفئران عن طريق الحقن باستثناء نترات الزنك عن طريق الفم لمدة ثلاثة أسابيع. تم تقدير متغيرات مختلفة مثل العلامات الدموية والكيميائية الحيوية و معايير الإجهاد التأكسدي . لوحظ ايضا التشريح المرضي للخصية وأنسجة الكبد. تم ايضا إجراء دراسة مخبرية و تحليل خصائص الجسيمات النانوية باستخدام بروتوكولات قياسية.

أثبتت نتائج الدراسة في المختبر تكوين الجسيمات النانوية بالطرق الكيميائية والبيولوجية ، مع أنشطة مهمة كمضادات لإنحلال الدم ومضادة للالتهابات. أظهرت نتائج الدراسة التي أجريت على الفئران أن الفئران المعالجة بالسيكلوفوسفاميد تعرضت لإضطراب في الخلايا المناعة والهيموجلوبين وخلايا الدم الحمراء والتستوستيرون والمعايير الكيميائية الحيوية مقارنة بالمجموعة الشاهدة . كما اضطربت أيضا معايير الإجهاد التأكسدي و المقاطع النسيجية للكبد و الخصية مقارنة بالفئران الشاهدة . أوضحت معاملة الفئران بعد السيكلوفوسفاميد بالزنك نتائج متباينة مع تفضيل الزنك المُصنَّع بالطريقة البيولوجية (حمض الأسكوربيك) ، حيث لاحظنا تحسنًا ملحوظًا في معظم المتغيرات السابقة. أشارت هذه الدراسة إلى أن الخاصية المضادة للالتهابات والمضادة للأكسدة AS-ZnONPs الجديدة سمحت باستخدامها لحماية الكبد والخصية من الآثار الجانبية للسيكلوفوسفاميد أو أدوية العلاج الكيميائي الأخرى .

الكلمات المفتاحية : السيكلوفوسفاميد , AA-ZnONPs , الخصية , الكبد , الإجهاد التأكسدي .

Abbreviations list

CPA : Cyclophosphamide

NPs : Nanoparticle

Zn : Zinc

ZnO : Zinc oxide

ZnONPs : Zinc oxide nanoparticle

AA : Ascorbic acid

KOH : Potassium hydroxide

AA-ZnONPs : Bio-synthesized nano-zinc oxide

K-ZnONPs : Chemically synthesized nano-zinc oxide

$Zn(NO_3)2$: Zinc salts

DNA : Deoxyribonucleic acid

RNA : Ribonucleic acid

CYP 450 : Cytochrome P 450

PM : Phosphoramide mustard

NM : Nitrogen mustard

Nu : Nucleophilic

ACRO : Acrolein

4-OH CPA : 4-hydroxycyclophosphamide

Aldo Cy : Aldophosphamide

GST : Glutathione S-transferase

ALDH: Aldehyde dehydrogenase

ADH: Alcohol dehydrogenase

ecto-CRT : Calreticulin of plasma membrane surface

T reg : Regulatory T cells

ATP : Adenosine triphosphate

NOR : Nornitrogen mustard

OS : Oxidative stress

ROS : Reactive species of oxygen

RNS : Reactive species of oxygen

RSS : Reactive species of sulfur

HO• : Hydroxyl radical

FR : Free radicals

H_2O_2 : Hydrogen peroxide

NADPH oxidase : Nicotinamide adenine dinucleotide phosphate

4- HNE : 4-hydroxynonenal

Anti oxi : Anti-oxidant

Oxi : Oxidant

LPO : Lipid peroxidation

MDA : Malondialdehyde

SOD : Superoxide dismutase

GSH : Glutathione

NO : Nitric oxide

$ONOO^-$: Peroxynitrite

NaCl : Sodium chloride

GOT : Glutamic oxaloacetic transaminase

GPT : Glutamic pyruvic transaminase

BSA : Bovine serum albumin

IC50 : Half-maximal inhibitory concentration

PBS : Phosphate-Buffered Saline

HCl : Chloride hydrogen

FTIR : Fourier Transform Infrared spectroscopy

UV/VIS : Visible/ultraviolet spectroscopy

SEM : Scanning electron microscope

Nrf2 : Nuclear factor erythroid 2-related factor 2

RBC : Red blood cells

WBC : White blood cells

Hb : Hemoglobin

3bHSD : 3b-hydroxysteroid dehydrogenase

17bHSD :17b-hydroxysteroid dehydrogenase

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Summary

Introduction

Introduction

Human growth is a basic phenomenon that depends mainly on cellular reproduction through division “mitosis” (Jonathan M. Scholey and al.,2003) . Where each mother cell gives two daughter cells (Joseph Y. Ong and al.,2019) . Under normal conditions the division is well controlled , and only healthy cells is maintained by the intervention of apoptosis (Hisae Tateishi-Karimata and al.,2021) . However, due to several reasons, this control is disturbed and cells divide excessively and abnormally “tumor” (Ma Hongbao and al.,2015) If the tumor has a high mitotic activity , invasive , necrotic ,ulcerative and Unlimited , It constitutes what is called “Cancer” (J.Xuereb and al.,2008) . Which is considered a dilemma because it occupies a large proportion of the causes of death (Fiorella Rossi and al.,2020) .

One of the methods used to treat cancer is chemotherapy, but it is often met with resistance by cancer cells (Ladislav Novotny and al.,2005) . Some types of chemotherapy rely on alkylating properties to control cancer cells and It mainly includes Cyclophosphamide “CPA” (Karol Bukowski and al.,2020218) . alkylating agents contain reactive alkyl groups “CnH2n+1” And by which it intervenes directly in the DNA and stops the division (Hannah Strobel and al.,2019) . On the other hand, long-term use of these agent , including CPA , causes side effects, including reduced sperm production in males and oxidative stress (Ranju Ralhan and al.,2007) which is associated with hepatotoxicity (Anup Ramachandran and al.,2018).

The body normally produced a free radical as a defense mechanisme contre harmful substances , on the other hand, antioxidants are produced to neutralize their harmful effects (Ahmed M Kabel.,2014) . Oxidative stress is an imbalance of antioxidants and oxidants in favor of the latter (Parham Taslimi and al.,2019322) . Oxidants “ free radicals ” are generated by several external “exposure to environmental pollutants and radiations , drug ” and internal factors “Immune cell activation, inflammation” (Gabriele Pizzino and al.,2017) . Oxidative stress plays a major role in the development of many diseases such as cancer, arthritis , autoimmune disorders, cardiovascular (Ashok Shinde and al.,2012) . Oxidative stress is also closely related to liver disease (Y. S. Voronkova and al., 2018) . Also , excessive generation of reactive oxygen species in the semen can cause the sperm to be destroyed (Marcello Cocuzzaand al.,2007) .

In view of these side pests of CPA , it is necessary to suggest a mechanism to combat them, Nowadays, the technology of reducing materials to nano-size “Nanotechnology” has

gained great interest due to the success in producing particles with positive properties (Amra Bratovcic .,2019) Attention has been paid to nanotechnology when it was proven that the size and shape of the material change its properties, for ex : by increasing the interaction ,This is very important in nanomedicine and biomedicine (Patrycja Paluszkiewicz and al.,2021) Despite the small size of the nanoparticles but have a large surface-tovolume ratio, which gives them phenomenal features (Abdul Waris and al.,2020) Besides the desired size and reaction speed, nanoparticles also enjoy easy passage through cellular barriers (Razieh Rezaei and al.,2019224) While the use of CPA produces side effects resulting from its effect on normal tissues, nanoparticles can improve the specificity of the drug (Patrick Boisseau and al.,2011)

Zinc is a trace element of medical and biological importance that participates in many biochemical reactions in the body and its deficiency is associated with several diseases, including hepatic and sexual diseases (Mohammad K. Mohommad and al.,2011, Ab Latif Wani and al.,2017) . There are five zinc compounds approved by the US Drug and Food Management, including zinc oxide nanoparticles “ZnONPs” (Sanodia Najoom and al.,2021) . In a recent study in 2019, it was concluded that the injection of these nanoparticles reduces testicular toxicity through its antioxidant activity (Zeynab Khamis El-Maddawy and al.,2019) . Another study in 2017, showed the possibility of inhibiting hepatic and renal fibrosis by ZnONPs by reducing the parameters of oxidative stress and inflammation (Samir A.E. Bashandy and al.,2017) . It should also be noted that these positive effects depend on the dose and that high doses can induce opposite effects (Said Said Elshama and al.,2018) .

Green synthesis of NPs is more positive compared to other methods , as the materials used during physical and chemical synthesis may reside in NPs formed , and this is toxic in medical application (Happy Agarwal and al.,2017) . The green synthesis is achieved by using plant extracts that have proven their worth compared to other green methods (Falak Thakral and al.,2021) . As another additional attempt to determine the exact reducing agent, green synthesis can be achieved by adding only ascorbic acid “Biological synthesis” (Natpasit Chaithanatkun and al.,2015) It is also an antioxidant for the benefit of liver function (Iffat Nayila .,2020) And dilute testicular toxicity (W.A. Oyeyemi and al.,2014) .

Based on the previous five points in which we proceeded from the seriousness of cancer to the lack of a clear treatment against it and the adoption of CPA , which has negative effects on the liver and testicles, down to the success achieved by ZnONPs and finally the possibility of

improving them by manufacturing them with ascorbic acid only , We have tried a treatment system against the negative effects of CPA based on AA-ZnONPs "Biological synthesis" compared to K-ZnONPs "chemical synthesis" and Zinc salt "zinc nitrate $Zn(NO_3)2$" , and we hope for its success.

After touching on two theoretical parts, to remind of some important points about chemotherapy, oxidative stress and nanotechnology. We presented the experimental part, which contains two parts :

- The first part: It is an in vitro study ; synthesis of ZnONPs , quantitative and qualitative characterization of these nanoparticles and evaluation of their biological property.
- The second part: It is an in vivo study ; Evaluation of the effects of therapeutic regimens on rats by conducting blood analyzes, oxidative stress criteria and histological analysis .

Theoretical part

Chapter 01:

Cyclophosphamide and oxidative stress

1. Cyclophosphamide "Chemotherapy"

1. 2. Definition and indication of cyclophosphamide

known as Cytoxan or Endoxan is a chemotherapeutic drug cytotoxic bifunctional alkylating agent that belongs to the oxazaphosphorine nitrogen mustard class. which is a non-active cyclic phosphamide ester of mechlorethamine . (Saif.S.Abd alhassan and al.,2018 ,Sung-Hwan Kim and al.,2013) (Neetu Singh and al.,2008 ,Hesham Farouk Hasan and al .,2020) . Its mechanism of action is to interfere with the replication of DNA and the creation of RNA (Gamal S. El Gharabawy and al. , 2019). CPA is widely used for treatment of various types of cancer diseases ; chronic lymphocytic leukemia, lymphomas, soft tissue and osteogenic sarcoma, and solid tumours (lung, breast, and ovary) (Yesi Ihdina Fityatal Hasanah and al., 2021). treat various autoimmune disorders such as systemic lupus erythematosus, multiple sclerosis (thetreatment of nephrotic syndrome and systemic lupuserythematosus In children (Kaian Amorim Teles and al., 2016)) and also used as an immunosuppressive - and a immunomodulatory abilities (Koji Kato and al.,2020) - drug for organ transplantation by suppression of cellular and humoral immunity through its actions on T cells and B cells (Sangita Singh and al.,2017 , Amer Awad and al .,2009).

1.3. Chemical structure

Cyclophosphamide (CPA) (2-[bis(2-chloroethyl)amino]-2H1,3,2-0xazaphosphorinane 2-oxide) Its chemical formula is C7H15Cl2N2O2P (Isabella L. Karle and al., 1977). Is a cyclic phosphoramide ester (Lucy H. Fraiser and al.,1991).

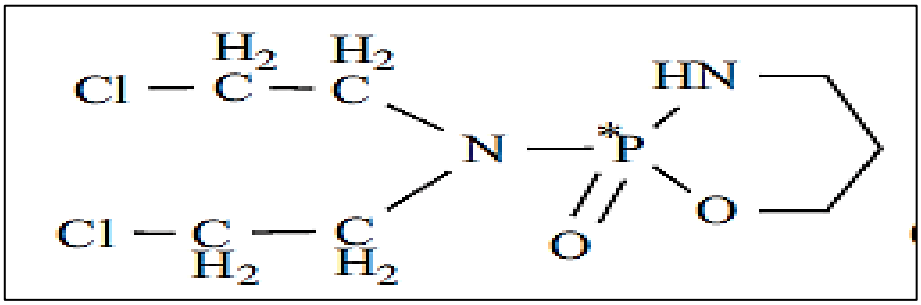

Figure 01 : Chemical structure of CPA (Jing Zhang and al.,2006)

1.4. Pharmacokinetics

Pharmacokinetics is used to study ADME : absorption, distribution, biotrasformations (metabolism) and elimination of drugs In the body after its administration , As a defect in one of the organ participating in these operations is common among critically ill patients (Derek J Roberts and al .,2013) (R. URSO and al.,2002).

About pharmacokinetics of cyclophosphamide , the polymorphisms in CYP 450 (CYP2B6) ; main enzyme in the metabolism process. It results in a change in some parameters such as : elimination half-life and clearance (Ibrahim El-Serafi and al.,2014)

1.4.1. Administration

Depending on the indication , the dose of the drug is determined well. Where cyclophosphamide is given in low doses to enhance immunity against tumors, and in high doses in the case of bone marrow transplants, for example, to suppress immunity (Ellyn Hughes and al.,2018 , Muluken Altaye Ayza and al.,2020)

Cyclophosphamide is administered orally “tablets and solutions (Javed Ahmad Khan and al.,2014)” or intravenously “for 10 to 60 minutes (Quan Tran and al.,2008)”, which a continuous oral administration causes less toxicity compared to intravenous administration of high doses with the same effect (anti-angiogenic effect in tumor cells) (Rachel Kennedy and al ., 2011)

1.4.2. Absorption

Cyclophosphamide is well absorbed after oral administration, with a bioavailability of greater than 75% (Z. FELEGARI.,2014) .

1.4.3. Distribution

CPA is distributed in the body through the blood and in the form of two metabolites “4hydroxycyclophosphamide and aldophosphamide” (F.D. JUMA and al.,1980).

1.4.4. Metabolism

The importance of cyclophosphamide metabolism is its activation - inactive per se - , Where it is metabolized in the liver (Hira L and al.,1981) . by hepatic microsomal cytochrome P450 (where the Changes in the level of expression of hepatic P-450 can affect the level of cyclophosphamide metabolism and accord ingly its efficacy (Gerald A. LeBlan and al.,1990)) mixed functional oxidase to generate two active metabolites "phosphoramide mustard and acrolein" (Bonsome Bokolo and al.,2018). CPA cytotoxic antitumor activities are associated with phosphoramide mustard (PM), whereas acrolein, responsible for its noxious side effects (Adio J. Akamo and al.,2021).

(Figure 01) The first step of metabolism is production of first metabolite4-hydroxycyclophosphamide (4-OH CPA) - that exists in equilibrium with its ring-opened tautomer aldophosphamide (Aldo Cy) - By oxidation of cyclophosphamide by Various human CYP 450 enzymes (Susanne Steinbrecht and al.,2020 . Shanthi Ganesan and al.,2017) . where these metabolites transported to tumour cells via the systemic circulation easier than ionic PM (Yi Luan and al .,2019[76], Gareth J. Veal and al.,2016) . Depending on cell type , Aldo Cy is may subjected to process β-elimination In the cytosol , for decompose to phosphoramide mustard (PM) and Acrolein (Koichiro Kurauchi and al.,2017, Marcin Włodarczyk and al .,2018) .

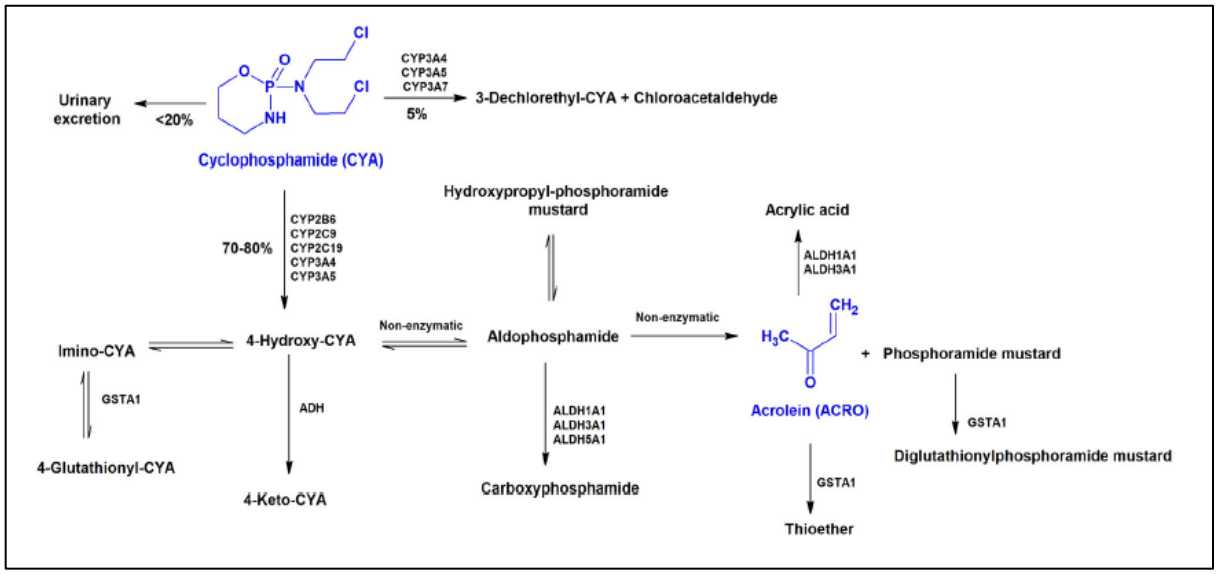

Figure 02 : Steps of CPA metabolism (Ana Reis-Mendes and al.,2019)

It is also possible through secondary paths : the dechloroacetylation of CPA results in the toxic agent chloroacetaldehyde , and the inactive 4-keto-CPA, 4- glutathionyl-CPA , carboxyphosphamide can be formed (C Rodriguez-Antona and al .,2006) where the last metabolite represents: a major stable non therapeutic metabolite of CPA found in clinical samples , GST : Glutathione S-transferase , ALDH: Aldehyde dehydrogenase , and ADH: Alcohol dehydrogenase ; They are the enzymes responsible for inhibiting the activity of CYP metabolites. This means detoxification of cyclophosphamide (Karolina Tecza and al .,2018 , Duan Wang and al .,2012)

1.4.5. Elimination

Cyclophosphamide is characterized by high Tubular reabsorption and by : elimination half-life about 6.5 hours (Monika Singh and al ., 2014). eliminated primarily via renal excretion , where the 20 % of cyclophosphamide unchanged and both the metabolitesis are excreted in urine Despite the high bioavailability (Shelby Barnett and al.,2021,Anna Carolina Batista Dantas and al.,2010 ,MARION HAUBITZ and al.,2002).

1.5. Mechanism of action

The mechanism of action of CPA varies according to the dose taken , Where low doses enhance the activity of the immune system against tumors (Figure 02) , On the other hand, high doses achieve cytotoxicity, whether tumor or lymphatic. And inhibits the therapeutic efficacy of the immune system by alkylating property (YASUHIDE MOTOYOSHI and al.,2006 , Rasha Abu Eid and al .,2016)

Among the effects of low dose CPA on immunity , M. E. Christine Lutsiak and al study in 2005 proved that low doses of CYP lead to inhibition of Treg cell functions in addition to reducing their number. also stimulates phagocytosis of the antigen and its presentation by dendritic cell (By enhancing tumor expression of ecto-CRT, and release of HMGB1 and ATP) .

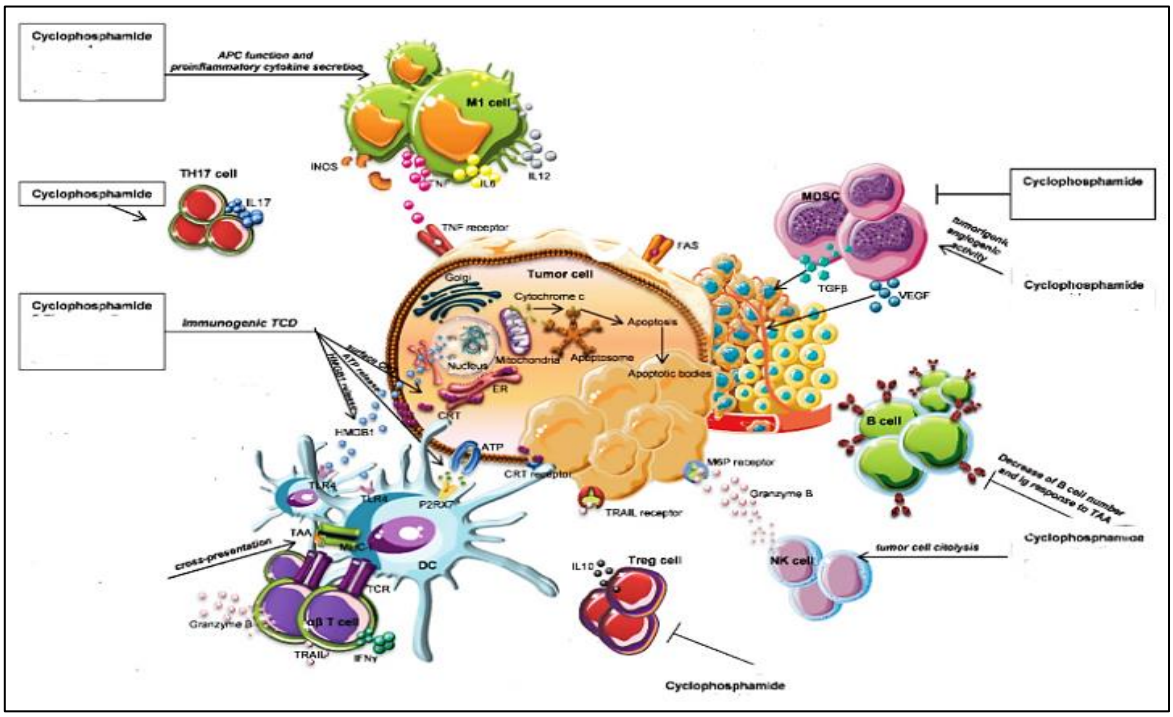

Figure 03 : The immune-modulatory effect of cyclophosphamide (low dose)

Alkylating agents interact directly with DNA, by abnormal pairing with bases, which leads to crosslinking of the DNA and eventually cell death (Ranju Ralhan and al.,2007). nitrogen mustards (meclorethamine) is The anti-cancer alkylating agents bearing the –N(CH2CH2Cl)2 moiety Clinically developed to give the drug the opportunity to reach cancer cells without previous interaction like CPA (inactive)(Subhendu Karmakar and al.,2016) .

After the metabolism process of cyclophosphamide, two active metabolite are generate : phosphoramide mustard (PM) ; responsible for the alkylation activity of CPA , and acrolein metabolite (a highly electrophilic, α,β-unsaturated aldehyde) a reactive aldehyde, is known to be the most toxic metabolite of CPA (Shanthi Ganesan and al.,2015 , Roohi Jeelani and al.,2017) . Under physiological conditions, PM : N,N-bis-(2-chloroethyl)-phosphorodiamidic acid can spontaneously dephosphoramidates to form nornitrogen mustard (NOR) = normeclorethamine It also has the property of alkylation (Arnold S. Groehler and al .,2016, Fredrik Lehmann and al .,2021) . PM is a directly active alkylating agent , which reacts through an aziridinium intermediate (Figure 03)(Kari Hemminki and al.,1987) .

and is the same mechanism of nitrogen mustard (Figure 04) , where PM is acts by causing a loss of two chlorine atoms (gradually) and the formation of a positively charged reactive aziridinium (alkylation) (Tarek M.K. Motawi and al.,2010) which reacts readily with bionucleophiles - nitrogen in the DNA - (Frauke Antoni and al .,2020)

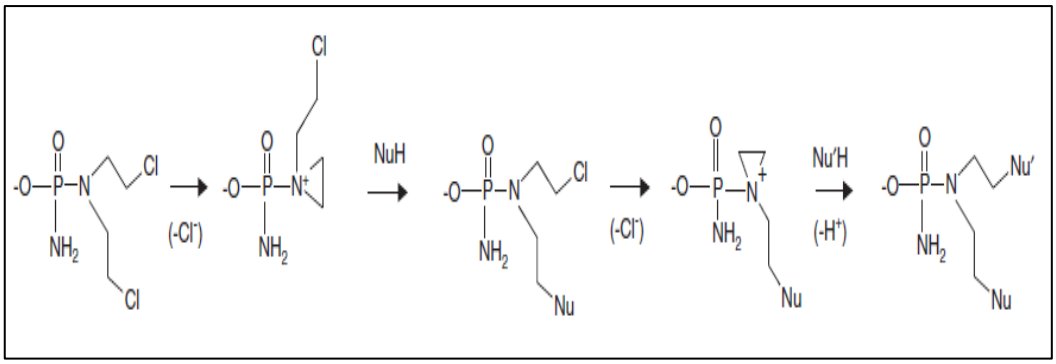

Figure 04 : Mechanism of binding of PM to an nucleophilic compound

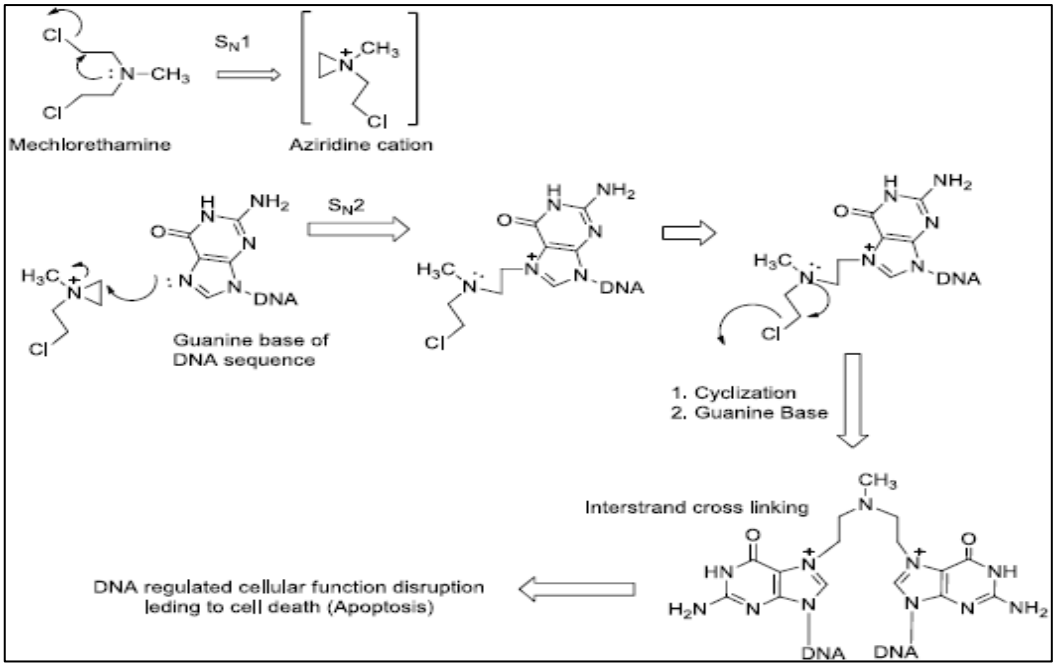

Figure 05 : Mechanism of NM alkylation of AND

The N-7 position of guanine in DNA is strongly nucleophilic and can be readily alkylated by the aziridium cation (The result of react the right and left arm) (Ashkan Emadi and al.,2009). leading to the formation of crosslinks in DNA strands at guanine N-7 positions (interstrand and intrastrand crosslinkages, respectively (Figure 05) (Anna González-Neira ,2012) Which leads to a stop in the vital processes in the cell and death in G2 and S phases of the cell cycle (Hydar muhsin khalfa and al .,2020) . The cell dies if the defect is not repaired, as the P53 protein is activated, which in turn leads to apoptosis (Georg Voelcker , 2020) .

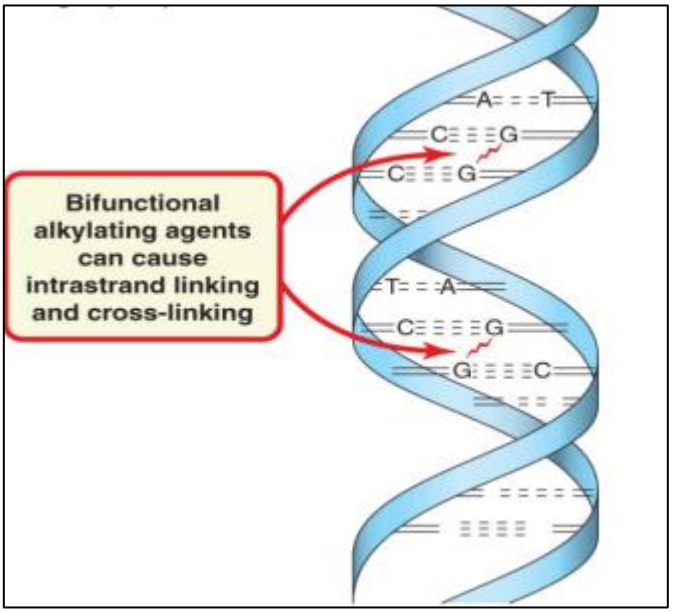

Figure 06 : Clarification the location of double alkylation of PM at the DNA

2. Oxidative Stress

2.1. Definition

Oxidative stress (OS) is defined as the biological phenomenon resulting from the superiority of oxidants over antioxidants " Figure01" (Helmut Sies and al.,2020) . and change oxidation-reduction (redox) state (Rhian M and al.,2020) . As a result of physiological or pathological conditions, which leads to disruption of redox signaling and control and/or cell damage (Munsoo Han and al.,2021) By damaging cellular macromolecules ; lipid , nucleic acid , protein (Almokhtar A Adwas and al.,2019) and carbohydrate through different mechanisms (OLGA BLOKHINA and al.,2002) Oxidative stress is linked to the development of many human diseases "Figure 02" , which gives it great importance for disease tracking. Like : hepatic (Derouiche and al.,2022) and Cardiovascular diseases (Thomas Senoner and al.,2019) Male reproductive system diseases as well (Paulina Nguyen-Powanda and al.,2020) .

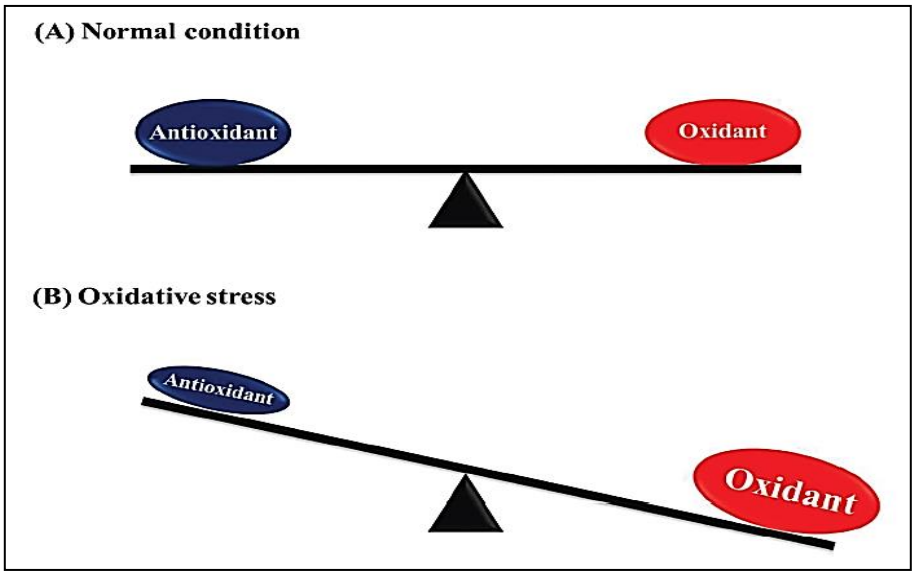

Figure 07 : A simple representation of the state of OS

(SARAWOOT PALIPOCH and al.,2015)

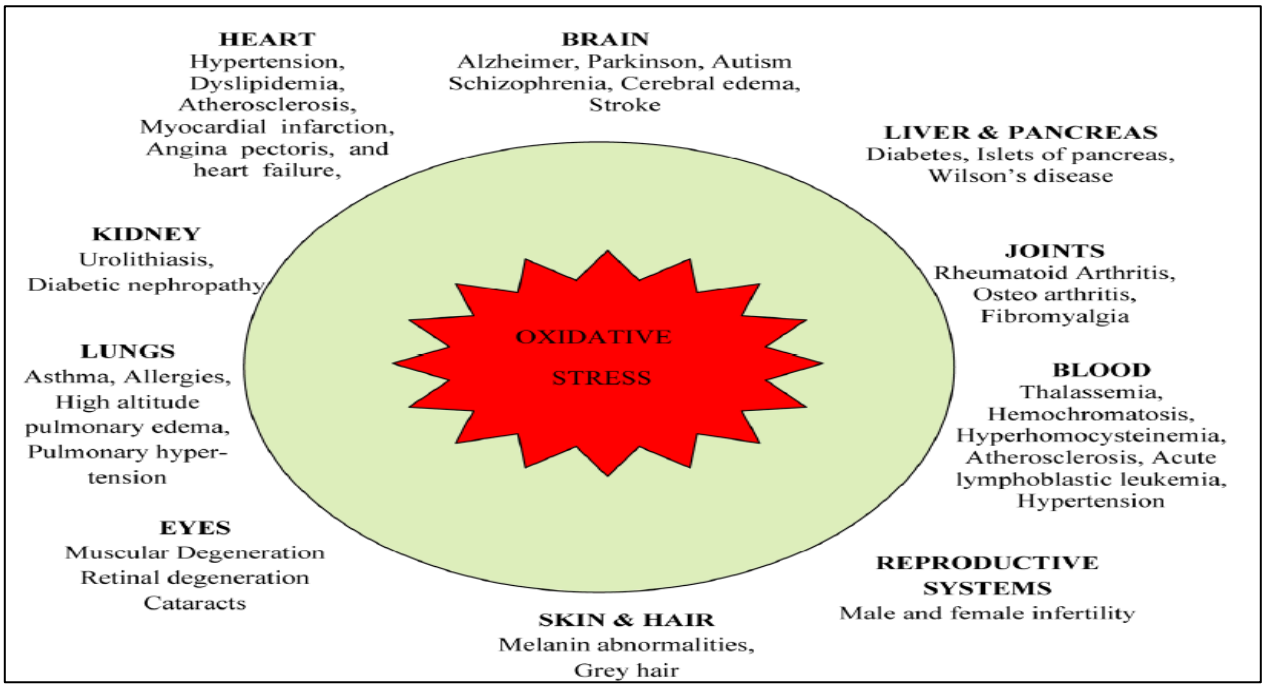

Figure 08 : Various disorders caused by oxidative stress to organs (Taibur Rahman and al.,2012)

2.2. Oxidants

They are reactive molecules that have the ability to oxidize various substrates in the body (Bruce N. Ames and al., 1993) including mainly : radicular and non-radicular Reactive species of oxygen "ROS", nitrogen "RNS" and sulfur "RSS" (Syed Saqib Ali and al.,2019) And because : RNS and RSS are derived depending on the first type "ROS" (Jian-Ming Lü and al.,2010) . ROS is the most important species (Yi-Jen Hsueh and al.,2022) . ROS include two Species : free radicals (FR) and non-radicals " Table 01 "(Lixiao Zhang and al.,2019) . Where this latter is also converted to free radicals (A.C. Georgiou and al.,2021) Which possesses a high ability to interact with various substrates (Mamta Sachdeva and al.,2014) .

Table 01 : Types of free radicals and non radicals ROS (Irina Georgiana Munteanu and al.,2021)

Reactive Oxygen Species		Non Free-Radical Species	
Hydroxyl radical	HO•	Hydrogen peroxide	H_2O_2
Superoxide radical	O_2•	Singlet oxygen	$^1O_2$
Hydroperoxyl radical	HOO•	Ozone	O_3
Lipid radical	L•	Lipid hydroperoxide	LOOH
Lipid peroxyl radical	LOO•	Hypochlorous acid	HOCl
Peroxyl radical	ROO•	Peroxynitrite	$ONOO^-$
Lipid alkoxyl radical	LO•	Dinitrogen trioxide	N_2O_3
Nitrogen dioxide radical	NO_2•	Nitrous acid	HNO_2
Nitric oxide radical	NO•	Nitryl chloride	NO_2Cl
Thiyl radical	RS•	Nitroxyl anion	NO^-
Protein radical	P•	Nitrosyl cation	NO^+

2.3. Free Radicals

Reactive species of oxygen : it is considered as a free radical or free radical producer (Dov Lichtenberg and al.,2015) . An atom in the normal state consists of a central nucleus surrounded by orbitals containing electrons (Toshikazu YOSHIKAWA and al.,2002) . But in the case of free radicals : An atom or molecule Unstable (capable of independent existence) has one or more unpaired electrons in its valence shell " Figure 03 " (Alugoju Phaniendra and al.,2014) . With high reactivity (for stability) and short half-life (M Irshad.,2002) . Where every cell in the human body is exposed to interaction with tens of thousands of free radicals daily (Majaz A. Qazi and al.,2018) . These radicals are formed (as a result of various factors) by the homolytic cleavage of the chemical bond from its molecules and by the intervention of redox reactions (Saikat Sen and al.,2010) . Free radicals are denoted by a bold dot on the side the chemical symbol (HO•), which indicates a unpaired electron (John M C Gutteridge and al.,1999).

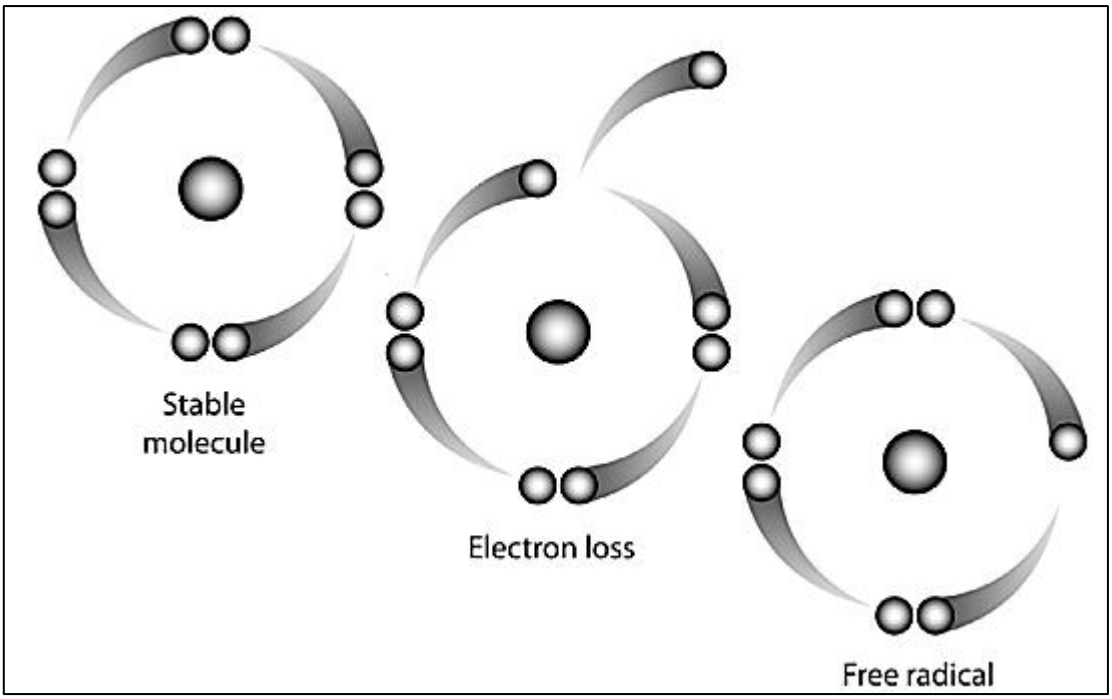

Figure 9 : Simple representation of the FR (Ashok Agarwal and al.,2008)

2.3.1. Free radicals Generating Factors:

Aerobic cells are produce FR continuously , where they act as secondary messengers in various intracellular signaling pathways (Xin Shi and al.,2022) . And as an agent of immune defense , Its production is accompanied by the production of antioxidants as well (Mehdi Sharifi-Rad and al.,2020) . its generation is depends on an exogen and endogen factors (Mohamed. G Khattab and al.,2022) .

2.3.1.1. Exogenous Factors :

FR can be generated after exposure to external factors such as : ionizing radiations, UV radiation , carcinogenic compounds, environmental pollutants " Figure 03 " (MIRAL DIZDAROGLU and al.,2012) . Also immunosuppressive treatments (Cyclophosphamide) used in cancer is a source of free radicals (Andrés García-Sánchez and al.,2020) .

2.3.1.2. Endogenous Factors :

Cellular enzymatic reactions are a factor in the production of FR , Therefore, the center of these reaction (organelles) is considered as an sources " Figure 03 " (V. Lobo and al.,2010) .

Mitochondria (most important) : During the reactions of the respiratory chain, approximately 1-2% of the oxygen is converted to the peroxide radical anion (Obeagu Emmanuel Ifeanyi 2018) . But with the intervention of antioxidants, it turns into H2O2 (non-radical), which allows for the possibility of a Fenton reaction and the production of hydroxyl radical "more active" (Anand Kumar Keshari and al .,2015) .

Other sources : peroxisomes also produce free radicals as a consequence of their normal metabolism (Luis A. del Rı´o and and al.,2016) . phagocytic cells " NADPH oxidase " (Mustafa Taha Mohammed and al.,2015) . enzymes of endoplasmic reticulum : cytochrome p-450 and b5 enzymes and diamine oxidase (Alugoju Phaniendra and al.,2014). FR can also generate from ionizing reactions "Non-enzymatic reactions" (Nisreen Husain and al.,2012) .

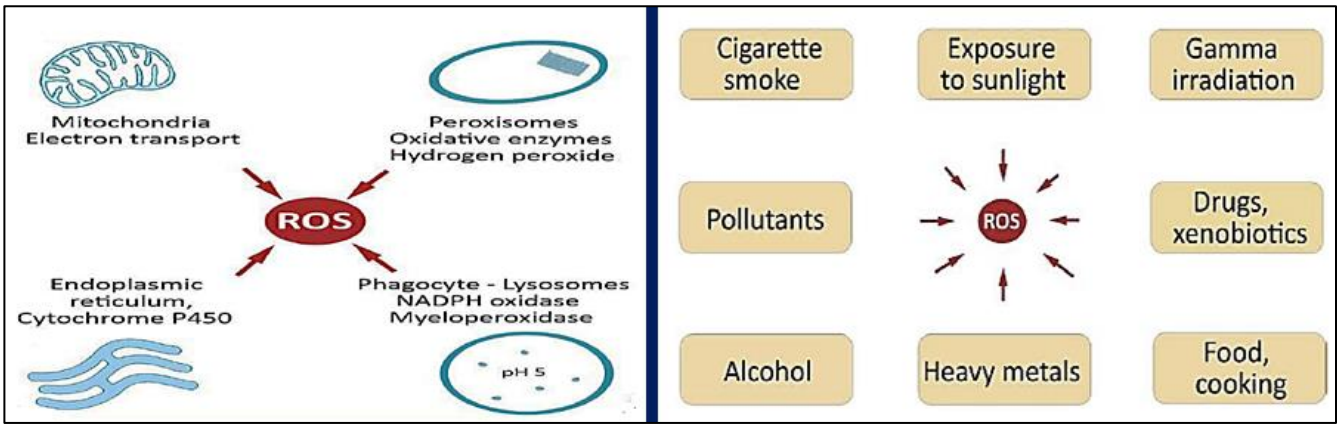

Figure 10 : Endogenous and Exogenous Factors of ROS generation (Arben Santo,2016)

2.3.2. Effects On Organism :

FR oxidize biomolecules to obtain an -e (stability), but the attacking molecules themselves turn into a FR after losing an -e , and thus the reactions continue until the intervention of antioxidants or the formation of a more stable FR (Abheri Das Sarma and al.,2010, Z.N.Kashmiri and al.,2014) .

As a result of this attack, free radicals are implicated in many bio molecular damages (Figure 04) :

- While Lipids are the main component of the cell membrane, they are the first goal to oxidation by FR "Lipid peroxidation" (Huiyong Yin and al.,2011) .LOOH , malondialdehyde (MDA) and 4-hydroxynonenal (4- HNE) : are the most important products , whereas 4-HNE is the most toxic (Antonio Ayala and al.,2014[158]) . while MDA is far the most popular indicator of oxidative damage to cells and tissues (Denise Grotto and al.,2008) .
- ROS can causes : DNA base alterations, strand breaks, One of the most abundant oxidative DNA lesions produced is 8-hydroxydeoxy guanosine , which is elevated in various human cancers (Saniya Arfin and al.,2021)
- FR can also oxidize protein, beginning by affecting the amino side chains (Saheem Ahmad and al.,2017) .
- On the other hand, FR are responsible for the production of pro-inflammatory molecules, which in turn play an important role in the development of diseases (Javier Checa and al.,2020) .

And because FR cause everything that was previously mentioned and more, there are molecules and enzymes dedicated to combating their toxicity in the body " Antioxidants " (M. Le Bras and al.,2005) .

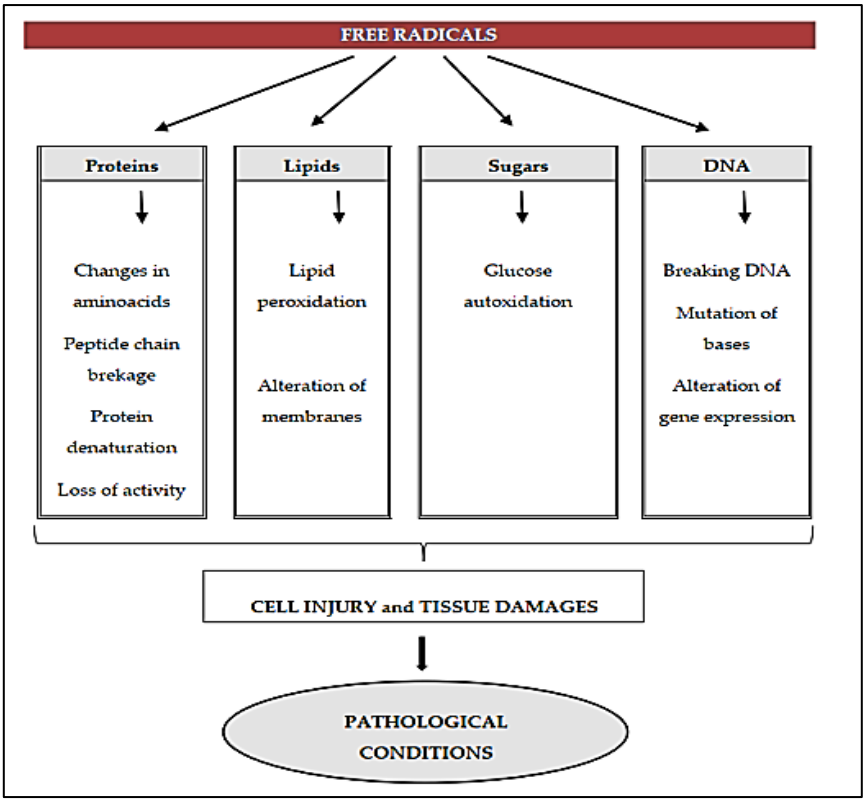

Figure 11 : Various effects of FR on bio macromolecules

(Giovanni Martemucci and al.,2022)

2.4 . Antioxidants :

Antioxidants are substances or agents that neutralize FR , block their generation or enhance endogenous antioxidants capabilities (Anu Shastri and al.,2016) . By donating an electron (Deepali Jat .,2016) . Oxidants differ in many ways, and this calls for different types of antioxidants to neutralize them (Márcio Carocho and al.,2012) . Antioxidants are divided on the basis of two criteria: their location (plasmatic : ascorbic acid , membranous : α- tocopherol , Intracellular : superoxide dismutase) (Manisha and al.,2017) . and their mechanism of action " Figure " , Enzymatic antioxidants : superoxide dismutases, catalases, and glutathione peroxidases (Emad A. Shalaby and al.,2013) . and non-enzymatic : Ascorbic acid , Beta-carotene (Ahmed M Kabel and al.,2014) . The major enzymes directly involved in the detoxification of ROS are superoxide dismutase "SOD" (A. T. Diplock and al.,1998) .

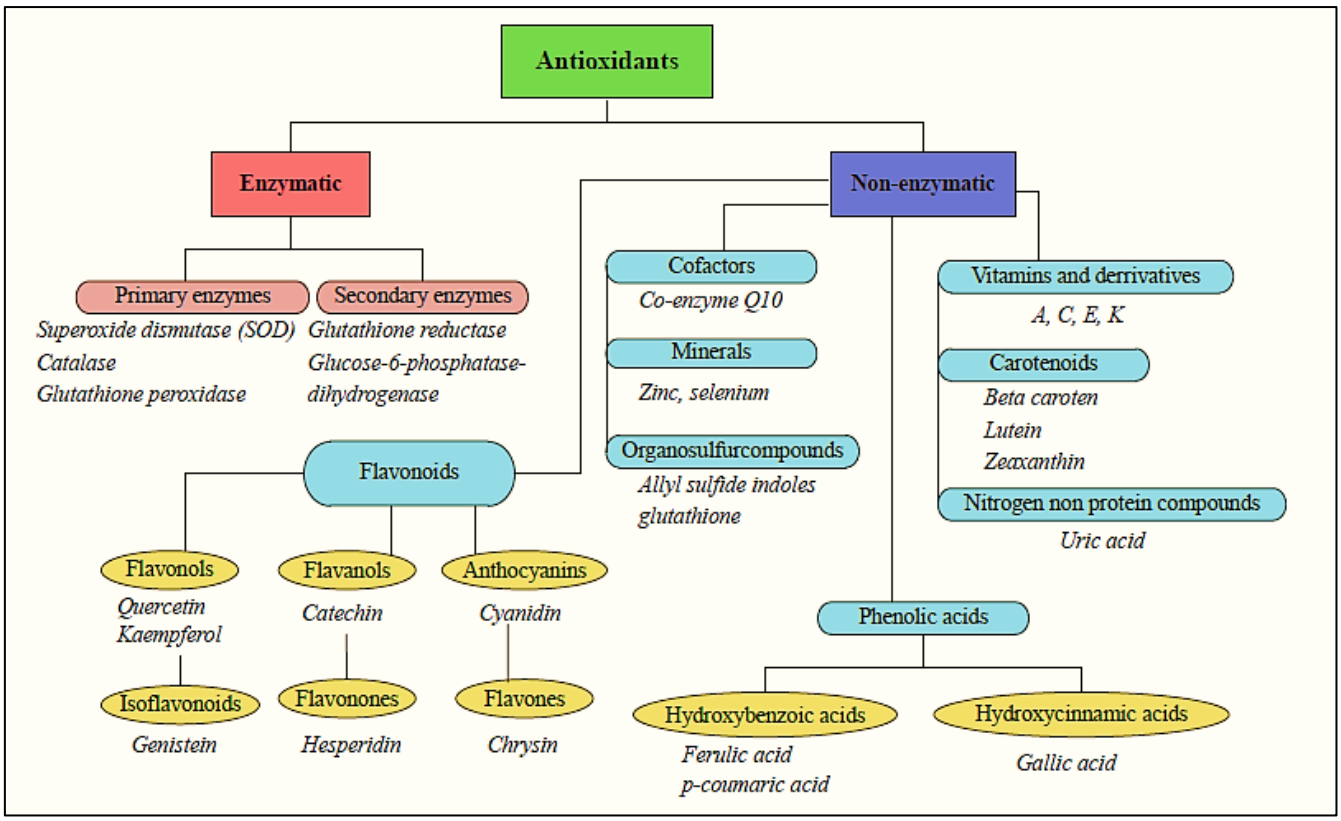

Figure 12 : Various types of antioxidants (Iryna Smetanska , 2018)

3. Cyclophosphamide and Oxidative Stress

Cyclophosphamide is an alkylating drug used in chemotherapy against various types of cancer and as an immunosuppressant (Pramita Chakraborty and al.,2014) . Although the main goal of CPA is to achieve the apoptosis of cancer cells, high-dose treatment achieved even toxicity to the rest of the tissues (A.H. ALHOWAIL and al.,2019) . Cyclophosphamide act to destroy antioxidants and from it the accumulation of ROS that contribute to its action on DNA (Jie Deng and al.,2018) . These radicals induce considerable structural damage irreversible , by interacting with proteins and DNA (Mohamed El-Shabrawy and al.,2020) . Cyclophosphamide is metabolized in the body into two metabolites : phosphoramide mustard “PM” and acrolein “ACR” (Cuneyt Caglayan and al.,2018) Acrolein is a metabolite associated with oxidative stress “ Figure” (Sultan Alqahtani and al.,2016) Where it reduce the levels of

antioxidants : glutathione “GSH”, catalase, and superoxide dismutase “SOD” (Shereen M. El kiki and al.,2020) Which leads to an increase ROS And causing: lipid peroxidation (LPO), protein carbonylation and oxidative DNA , despite the short biological half-life of ACR (Saleem H. Aladaileh and al.,2019) .

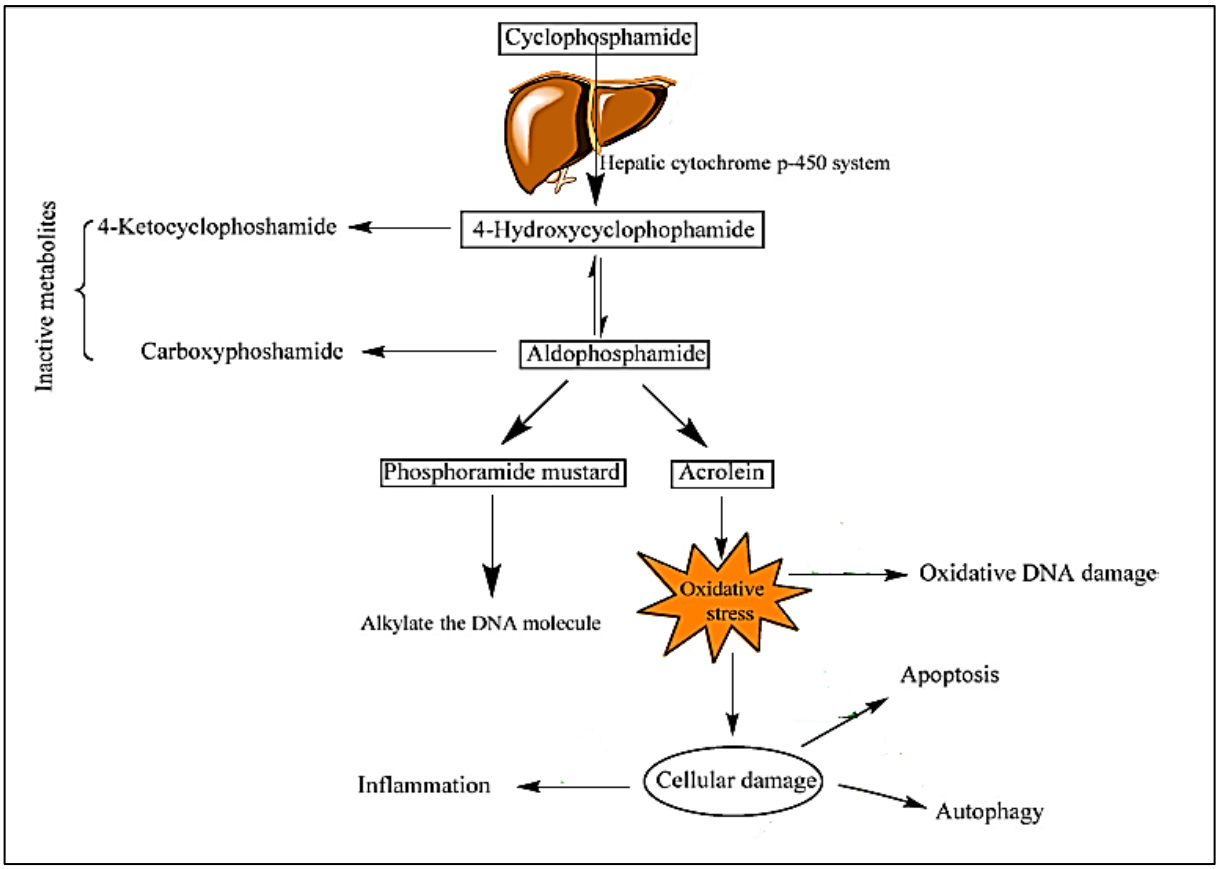

Figure 13 : From CPA administration to OS (Cuneyt Caglayan and al.,2018)

Chapter 02 :

Nanotechnology and Zn oxide nanoparticle

1. Nanotechnology

The origin of nanotechnology dates back to 1958, after which it developed through stages, reaching molecular nanotechnology in 2011 (Anna Pratima Nikalje and al.,2015) . In short, it means: the science that deals with particles and controls them. with dimensions ranging from 1 to 100 nano, which gives them new properties and functions (JE Hulla and al.,2015) . Nanotechnology has contributed to the development of sectors such as: medicine (nanomedicine), and life sciences (nanobiotechnologies) (Shariat Mobasser and al.,2016, Ludovica Lorusso ,2013) . Despite the tremendous strength of this technology products and its superiority over ordinary products, it faces real challenges in terms of its toxicity (Suprava Pate and al.,2015) .

2. Nanoparticles "NPs"

They are particles that have a nanometer in size , A nanometer is one billionth of a meter (10^9 m) about a thousand times smaller than a red blood cell (C. Chinglenthoiba and al.,2017) . and It belongs to the same domain as ultrafine particles (airborne particles) and places it as a subgroup of colloidal particles (P. Christian and al.,2008).Nanoparticles have several properties, including: particle size, structure, density and surface intrinsic reactivity (.Eka Sri Yusmartini and al.,2015). Depending on these properties, nanoparticles have gained several advantages : more personalized, portable, cheaper, safer, and easier to administer (Saba Hasan and al .,2015) . Nanoparticles have many applications, the most important of which is: nanomedicine , Where it has been applied against many diseases, including cancer (Carmen Paus and al.,2021) .

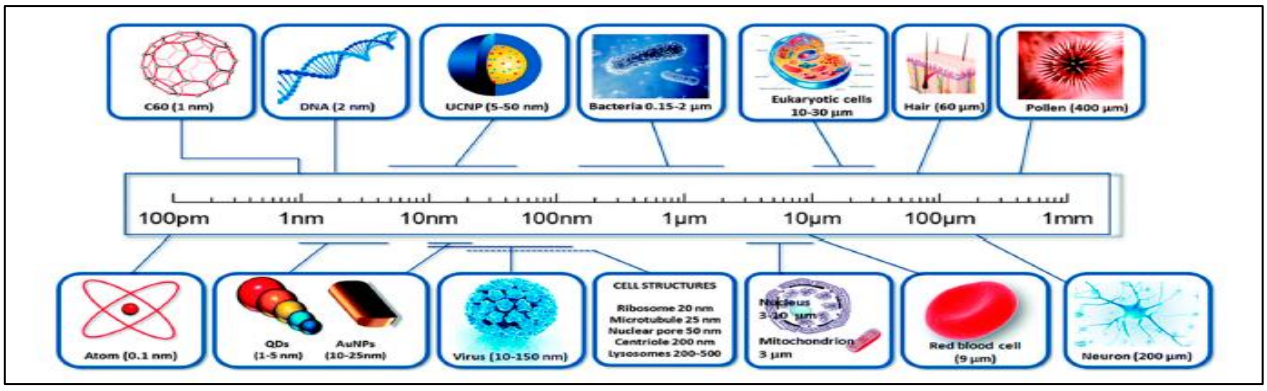

Figure 14 : Nano-sized particles compared to others that are smaller and bigger (Samer Bayda and al.,2019)

2.1. Classification of nanoparticles

Nanoparticles can be divided according to their nature into two main types “Figure” (Hussain H. Al-Kayiem and al.,2013) .

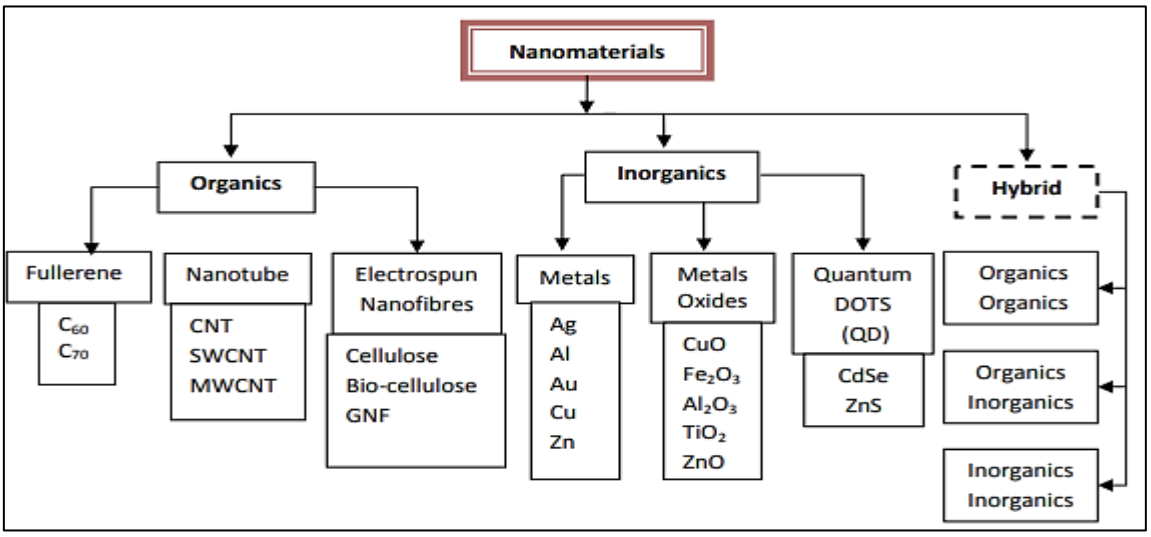

Figure 15 : Classification of NPs (Hussain H. Al-Kayiem and al.,2013)

At the nanomedicine level : metal oxide NPs “ZnO for ex” have many advantages such as high stability , geometry of shape, size and porosity is appropriate , easy incorporation into hydrophobic and hydrophilic systems (Maria P. Nikolova and al.,2020) .

2.1. Synthesis of nanoparticles

Nanoparticles can be synthesized by three ways : physical, chemical and green methods (K. Vithiya and al.,2011) . These synthesis methods are very important because they determine the physical and chemical properties of nanoparticles, on the basis of which the human body reacts with them (Anuradha Yadav.,2017) . As a result of the high costs of chemical and physical methods and their varying toxicity, the green synthesis was resorted to (Tejaswi Thunugunta and al.,2015) . Where the biomolecules act as reducing agents and the Synthesis of nanoparticles occurs under certain conditions (Khwaja Salahuddin Siddiqi and al.,2016) . Green synthesis is considered easy, more convenient and environmentally friendly (Hayrunnisa NADAROĞLU and al.,2017). Since the advent of nanotechnology, a solvent-based synthesis system has been used,

whereby water is the best solvent and works on molecular oxidation of nanoparticles because it contains oxygen (Jagpreet Singh and al.,2018) . The green method can be applied by using green reducing agents of biological origin such as : Ascorbic acid (Shrikant R and al.,2011) .

3. Zinc oxide nanoparticles "ZnONPs"

Zinc is a essential trace elements in human body , It is associated with the activity of 300 enzymes, and its deficiency is considered a disaster in the body (Debjit Bhowmik and al.,2010) . Zinc is supplied from external sources, the zinc nanoparticles provide relatively wider surface areas than the bulk ones (Asmaa H. Hammadi and al.,2020) . Zinc oxide "ZnO" is an inorganic compound and insoluble in water (Sidra Sabir and al.,2014) . and non toxic , It has many applications in different fields (Mohd Fadhlan Shah Hermandy and al.,2020) . Zinc oxide nanoparticles "ZnONPs" were also shown unique properties such as: low toxicity, high selectivity and biocompatibility (Melika Mohammadian and al.,2018) . Some studies also showed the possibility of reducing oxidative stress levels by ZnONPs And its anti-inflammatory activity (Samir A.E. Bashandy and al.,2017) . Other studies also demonstrated its antioxidant activity against doxorubicin-induced male reproductive toxicity (Puran Badkoobeh and al.,2013) .

Experimental Part

Chapter 3: Material and methods

1. Materials

1.2. Animal Materials

The experiment was conducted on 25 adult male rats , weighing between 150-250 grams at the beginning of the experiment. they were taken care of in a designated place at the level of the Faculty of Natural Sciences and Life at the University of EL-Chahid Hamma Lakhdar El Oued , Algeria .

1.2.1. Rat Breeding Circumstances

The rats were cared at a constant low temperature almost throughout the experiment (16 ± 2) And low humidity as well . And under normal light conditions . And to ensure the comfort of the mouse, only five rats were raised in laboratory plastic cages with easy access to water and food (corn powder) , Food was also placed in glass containers . And to ensure clean living, sawdust was placed in the cages and changed each period . In addition to weighing them weekly.

1.2.2. Groups Of Rats With The Treatment Regimen

After adaptation of rats to the conditions of the place for a period of ten days , They were distributed into five groups, similar in terms of weight average .

- ***Group 1 (control):*** It consisted of only five control rats that received intraperitoneal doses of physiological solution (0.9 % NaCl) , Parallel to the injection of the other groups **.**
- ***Group 2* (**CPA**):** They were injected with cyclophosphamide (30 mg / Kg b.w.) for three consecutive days + Injection with physiological solution in parallel with the injection of the rest of the groups
- **Group 3 (**CPA+**AS-ZnNPs)*:*** They were injected with cyclophosphamide + Intraperitoneal injection of AS-ZnOPs (Biological synthesis) at 7 mg / Kg b.w./week On the fourth day after Cyclo injection , Twice a week for three weeks .
- **Group 4 (**CPA+**K-ZnNPs)*:*** They were injected with cyclophosphamide + Intraperitoneal injection of K-ZnOPs (Chemical synthesis) : 7 mg / Kg b.w./week On the fourth day after Cyclo injection , Twice a week for three weeks

- ***Group* 5 (**CPA+**Zn(NO$_3$)2):** They were injected with cyclophosphamide + Orally Zinc salt (Zinc Nitrate) ; (54 mg/kg b.w.) + Injection with physiological solution in parallel with the injection of the rest of the groups .

1.2.5. Sacrefice and collecting the blood and organs

After the expiration of thirty-five days and the end of the experiment , The ratd were fasted by removing water, food and sawdust on them for ten hours . in The next morning day , They are sacrificed (by decapitation) After they were anesthetized by chloroform (94%) In a closed box for a few seconds To facilitate dealing with the mouse during sacrifice . The rest of the steps are explained in the following experimental design .

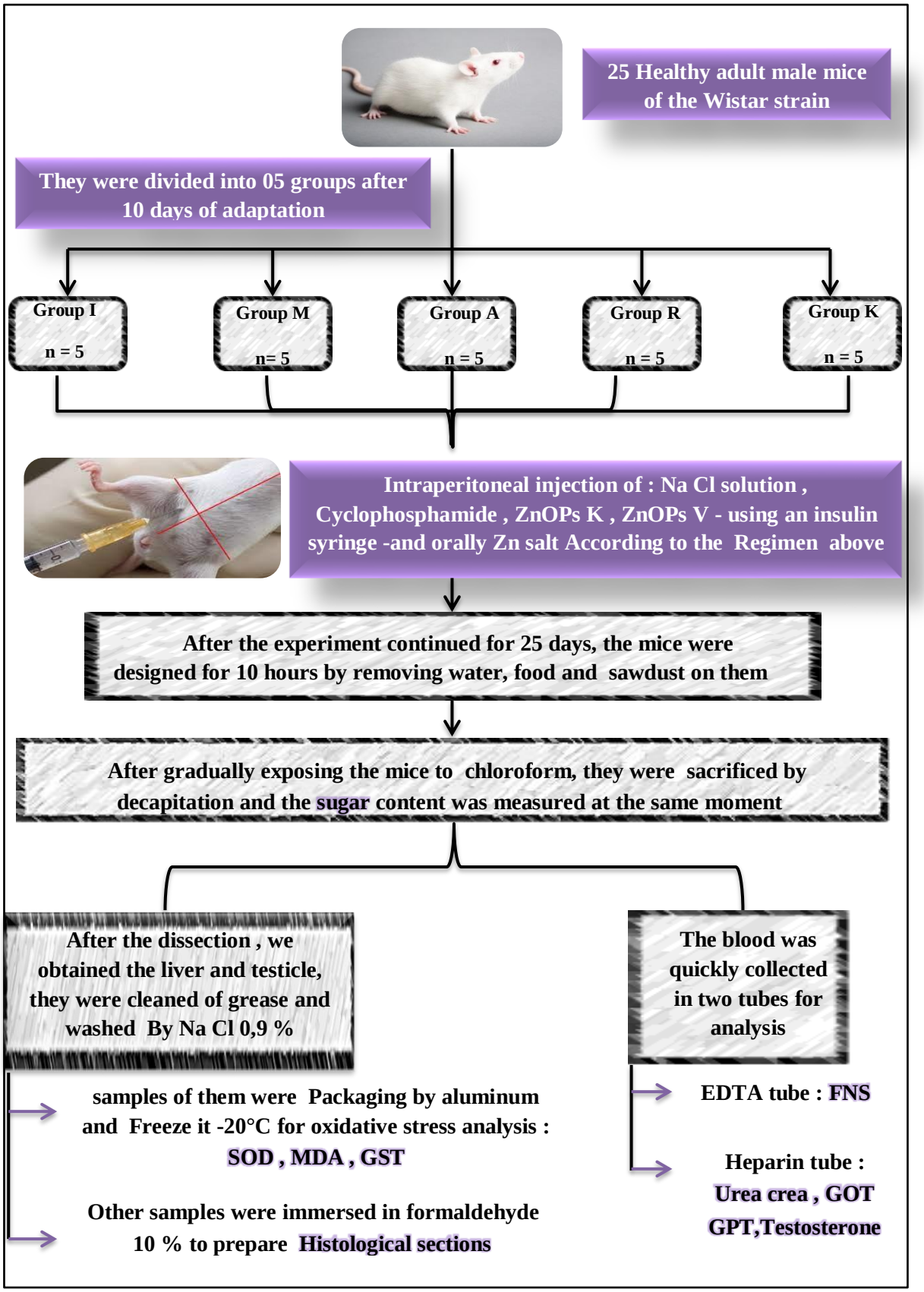

Figure 16 : Summary design of the experimental protocol

1.3. Reagents and products

Zinc nitrate ($Zn(NO_3)_2$, zinc sulphate ($ZnSO_4$) , Hydrogen chloride (HCl) , chloroform, sodium chloride (NaCl), Potassium hydroxide (KOH) , ascorbic acid (C6H8O6) , Tris (C4H11NO3) , Riboflavin , 1-chloro 2,4- dinitrobenzene (CDNB), glutathione (GSH) Trichloroacetic Acid (TCA), Thiobarbituric Acid (TBA), Butylated Hydroxy Toluene (BHT), nitroblue tetrazolium (NBT) , Bovine serum albumin (BSA) , Dipotassium phosphate (K2HPO4) , Potassium dihydrogen phosphate (KH_2PO4) , potassium diclofenac , Ethanol, Ethylenediaminetetraacetic acid (EDTA) , physiological solution (Na Cl 0.9 %) , Distilled water .

2. Methods

2.1. In vitro study

2.1.1. Synthesis of ZnNOPs

2.1.1.1. Biological synthesis of ZnONPs (AA-ZnONPs)

In order to synthesize the ZnO nanoparticle, 7.04 g Ascorbic acid was dissolved in 100 mL distilled water. After then 7.04 g Zn (NO3)2.6H2O is added to 200ml distilled water. Ascorbic acid solution is added drop wise to Zn-salt solution with continuous stirring. The solution was kept for overnight for precipitation. Precipitation was then centrifuged three times at 6000 rpm for 25 min to ensure the complete separation of nanoparticles. Centrifuged nanoparticles were then air dried in an oven at 105 °C for three hours and washed in double-distilled water and ethanol, respectively (Ahammed and al.,2020). The steps are explained more in the following design .

2.1.1.2. Chemical synthesis of ZnONPs (K-ZnOPs)

ZnO nanoparticles were synthesized by direct precipitation method using zinc nitrate and KOH as precursors. In this work, the aqueous solution (0.2 M) of zinc nitrate (Zn(NO3)2.6H2O) and the solution (0.4 M) of KOH were prepared with deionized water, respectively. The KOH solution was slowly added into zinc nitrate solution at room temperature under vigorous stirring, which resulted in the formation of a white suspension. The white product was centrifuged at 5000 rpm for 20 min and washed three times with distilled water, and washed with absolute alcohol at last. The obtained product was calcined at 500 °C in air atmosphere for 3 hr (Ghorbani and al.,2015) The steps are explained more in the following design .

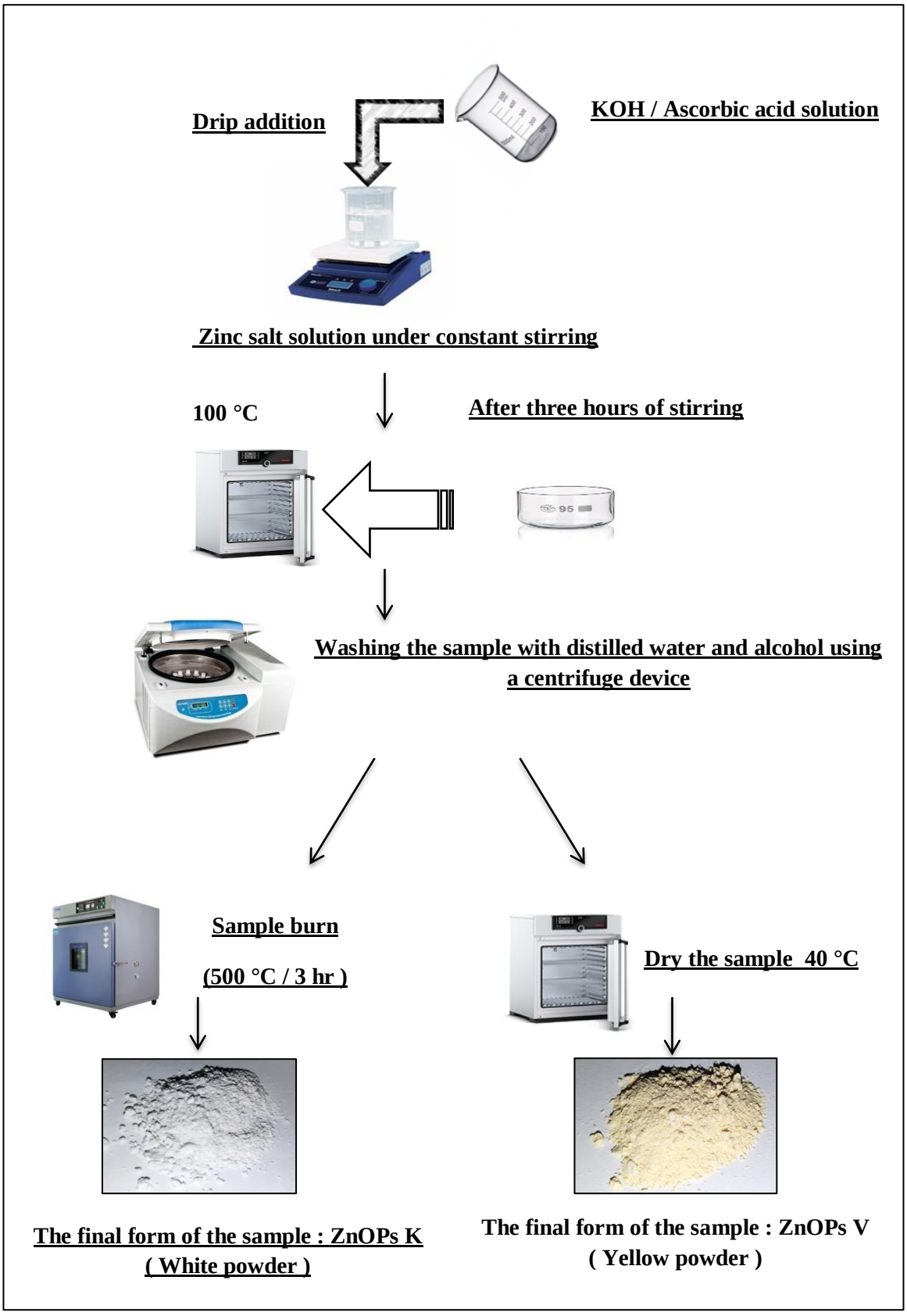

Figure 17 : Summary design of Synthesis of ZnONPs

2.1.1.3. Characterization of ZnONPs

Some characteristics of the two samples : ZnONPs V and ZnONPs K were determined by their analysis by : Fourier transform infrared spectroscopy and Scanning electron microscope . Where the first provides identification of the vital components of the samples (Ahmed Fadlelmoula and al.,2022[178]) . whereas The second technique allows to identify the morphology (James A. Seyforth ., 2015[179]) .

2.1.2. Biological activity tests of ZnONPs

Various biological tests were performed on the samples, including: anti-inflammatory properties tested , Hemolysis assay .

2.1.2.1. Anti-inflammatory activity

- **Principle**

Protein denaturation is associated with the formation of inflammatory disorders (Sarveswaran R and al.,2017[180]) . For this reason The anti-inflammatory activity is measured of protein denaturation inhibition in presence of the anti-inflammatory compound, which is studied through in vitro assay. The measured turbidity at 660 nm is proportional to the concentration of anti-inflammatory compound present in the sample (Vennila and al., 2018).

- **Procedure**

1. Add different concentrations (10–50 µg ml-1) of the sample / Diclofenac (standard) to bovine serum albumin (BSA) solution (1%) .

2. Incubation during 30 min at room temperature.

3. The pH of the solution was adjusted to 2 using dropwise addition of concentrated HCl.

4. After incubation, the mixture is heated at 72 °C for 30 min.

5. The all tubes are cooled for 10 min.

6. The turbidity is measured at a wavelength of 660 nm.

- **Expression of results**

The results are expressed by inhibition percentage (IP) of protein denaturation is calculated as follows :

$$IP\ (\%) = ((A\ sample - A\ control) / A\ sample) \times 100$$

Then the results are expressed by IC50 .

2.1.2.2. Hemolysis assay

- **Principle**

The Hemolysis assay is done as described by (Vinjamuri., and al 2015) that determined the protective effect of the antioxidant compound presented in the sample against the membrane erythrocyte lysis which induced by 1X PBS. The detection of membrane RBCs lysis by measuring the concentration of hemoglobin in blood plasma at 540 nm by spectrophotometer.

- **Procedure**

1. 5mL of blood was collected from healthy volunteers in the tubes containing 5.4 mg of EDTA to prevent coagulation.

2. The blood centrifuged at 1000 rpm for 10 min at 40C.

3. Plasma is removed carefully and the white buffy layer was completely removed by aspiration with a pipette with utmost care.

4. The erythrocytes were then washed for additional three times with 1X PBS, pH 7.4 for 5 min.

5. The Washed erythrocytes were stored at 4oC and used within 6 h for the hemolysis assay.

6. Add 50 µL of 10 dilutions (100 µL Erythrocytes suspension and 900 µL 1XPBS) of erythrocytes suspension was mixed with 100 µL of test samples (20-80ng/mL), 100 µL of 1XPBS was used as a control.

7. Reaction mixture is incubated at 370C water bath for 60 min.

8. The volume of reaction mixture is made up to 1 mL by adding 850 µL of 1XPB.

9. The reaction mixture is centrifuged at 300rpm for 3min

10. The resulting hemoglobin in supernatant is measured at 540 nm by spectrophotometer to determine the concentration of hemoglobin.

- **Expression of results**

The results are expressed by Hemolysis inhibition percentage and is calculated as follows :

$$IP\ (\%) = 100-(OD\ sample) / (OD\ control)\times 100$$

2.2. In vivo study

2.2.1. Hematological analysis

Hematological parameters were determined automatically using a Rayto machine.

2.2.2. Biochemical analysis

Biochemical parameters were determined by automatically using a spectrophotometer Kenya Max biochemistry and BIOSCAN reagent . but , hormonal parameters were determined by automatically using a Cobas-e411 and Roche reagent.

2.2.3. Oxidative stress tests

2.2.3.1. Homogenates preparation

One gram of (testicle and liver) tissue of all experimental group was grinding and homogenized in 9ml of buffer solution of Tris buffer saline (TBS, pH=7.4). Homogenates were centrifuged at (5000 rpm, 15min) , and the obtained supernatant was conserved at -20°C .

2.2.3.2. Determination of malondialdehyde (MDA) level

- **Principle**

The common method for the assessment of MDA level is thiobarbituric acid (TBA) assay, Where MDA form a complex with two molecules of 2-thiobarbituric acid (TBA) In the presence of acidic medium and heat. A change in the color of solution to a pink color is an indication of the presence of MDA (Prima Nanda Fauziah and al.,2018[181]) .

- **Procedure**

375mg of TBA, 20g of TCA, 0.01g of BHT, 25ml of 1N HCL and 50ml of distilled water were introduced into a beaker. The solution obtained was heated to 40° C. in a water bath until the TBA was completely dissolved, then transferred to a 100 ml flask and the volume filled up with distilled water to the gauge.

- **Expression of results**

The concentration of TBARS was determined using the molecular extinction coefficient of MDA (å= 1.53 105 M-1 cm-1). The results were expressed in µmol/1.

MDA (µmol/mg of prot) = (OD sample /1.53 x 10^5) / mg of protein

2.2.3.3. Determination of Super Oxide Dismutase (SOD) activity

- **Principle**

This test is based on inhibition of NBT reduction by SOD . NBT is reduced by superoxide anion O_2^- . and it is known that SOD neutralizes O_2^-. What inhibit the reduction of NBT (Christian AUCLAIR and al.,1978) .

- **Procedure**

Table 02 : Procedure of SOD test

Collect in tubes	Blank	Sample
EDTA-Met (0.1mM, 13mM)	1000µL	1000µL
Phosphate buffer (50Mm)	1892,2µL	1842,2µL
Sample	-	50
NBT (75µM)	85,2µL	85,2µL
Riboflavin (2µM)	22,6µL	22,6µL

- **Expression of results**

The results are expressed by Inhibition percentage of NBT reduction by SOD

IP (%) = (OD blank – OD sample) / (OD blank)×100

2.2.2.4. Determination Glutathione-S-transferase (GST) activity

- **Principle**

This test is based on measure the rate of conjugation of GHS to CDNB , What expresses the activity of GST (Positive relationship) , This requires a spectrophotometer at 340 nm . also , The results can be determined visually from the color change (John G. Vontas and al.,2000) .

- **Procedure**

Table 03 : Procedure of GST test

Reagents	Blank (µl)	Assay (µl)
Phosphate buffer (0.1M) ph 6.5	850	830
CDNB (0.02M)	50	50
GSH (0.1M)	100	100
Homogenate	-	20

- **Expression of results**

The activity of the GST expressed in nanomoles of CDNB per minute per milligram of proteins (nmol CDNB / min / mg prot) according to the following formula :

GST (nM/min/mg of prot)=(OD sample/min-OD blank/min)/(9.6×mg of prot)

2.2.3. Histological study

After immersing a party of the testicle and liver in formaldehyde, at least for a whole day, They were passed inside the drying machine "SLEE MTP "for 16 hours (twelve containers, starting with ethanol, passing through xylene, and ending with paraffin) . then steps of inclusion, Then samples were obtained with a width of three micrometer By the microtome machine. Then we got rid of the paraffin by immersing the samples in a water bath 45° . Then we entered the samples in the oven 70° . After the samples were dried, they were stained and observed with a microscope (OPTIKA) .

2.2.4. statistical analysis

Excel and Word (2010) were used to express the results in the form: Tables, Curves, Columns. Other results have been expressed as an average plus or minus the average standard deviation (Avg ± SEM). Statistical analysis of the data was carried out using the MINITAB software (Version 13). Where the results are translated to - given that the value p is the Significance threshold- :

-Significant when : $P < 0.05$

-Highly significant when $P < 0.01$

-Very highly significant when $P < 0.001$

Chapter II:

Results

1. Results of vitro study

1.1. Characterization of ZnONPs

1.1.1. AA-ZnONPs

1.1.1.1. UV/Vis analysis

UV-Vis absorption spectrum of ZnONPs synthesized by ascorbic acid is shown in (Figure 18) . Where several peacks between wavelengths 200 and 290 have been determined, which enables us to prove the formation of nanoparticles by the appearance of these distinctive peacks.

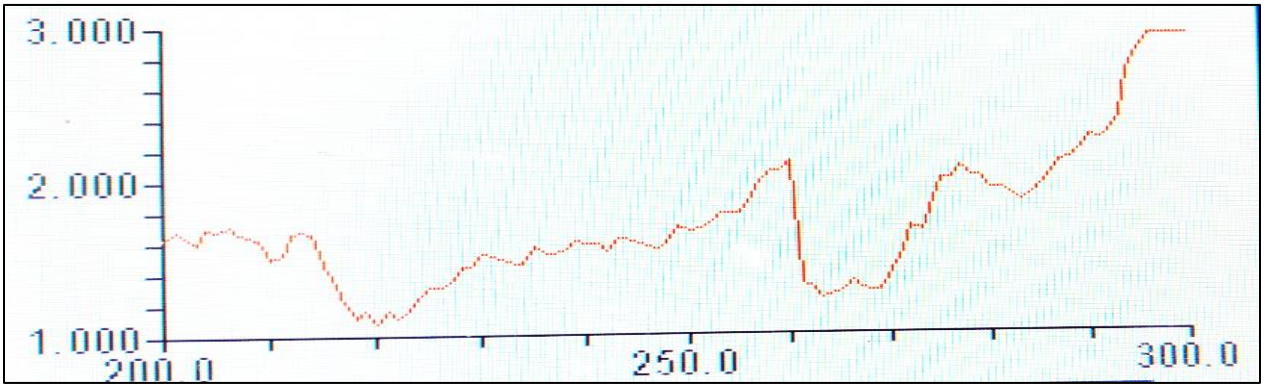

Figure 18 : UV-Vis spectrum of AA-ZnONPs

1.1.1.2. FTIR analysis

IR absorption spectrum of ZnONPs V is shown in (Figure 19) , as the emergence of a peak near 700 directly indicates Zn-O .

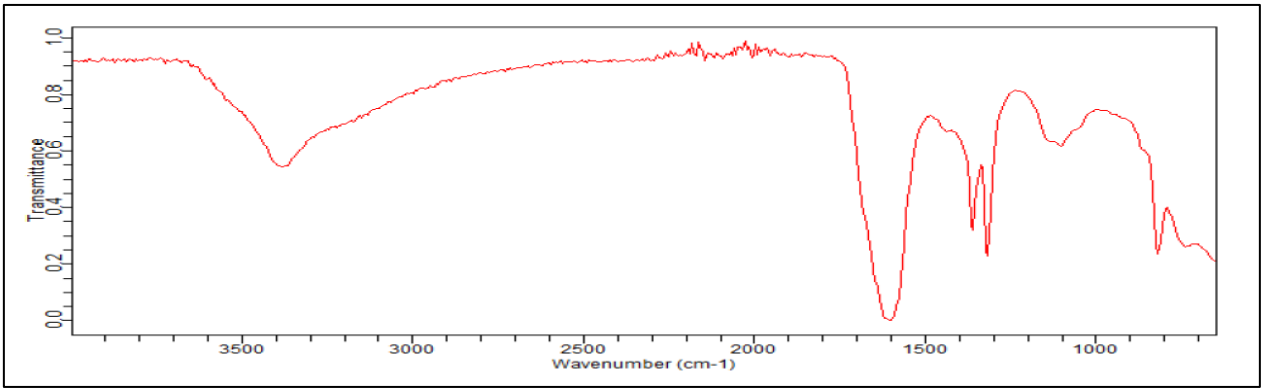

Figure 19 : Infrared spectrum of AA-ZnONPs

1.1.2. ZnONPs K

1.1.2.1. UV/Vis analysis

UV-Vis absorption spectrum of ZnONPs synthesized by KOH is shown in (Figure 20) . Where several peacks between wavelengths 200 and 290 have been determined, which enables us to prove the formation of nanoparticles by the appearance of these distinctive peackets. .

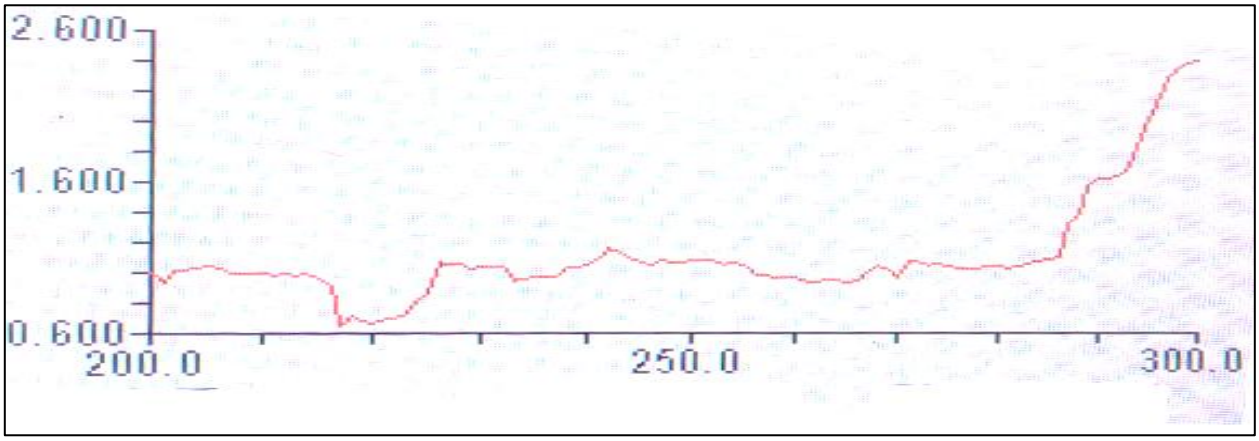

Figure 20 : UV-Vis spectrum of ZnONPs K

1.1.2.2. FTIR analysis

IR absorption spectrum of ZnONPs K is shown in (Figure 21) , as the emergence of a peak near 700 directly indicates Zn-O

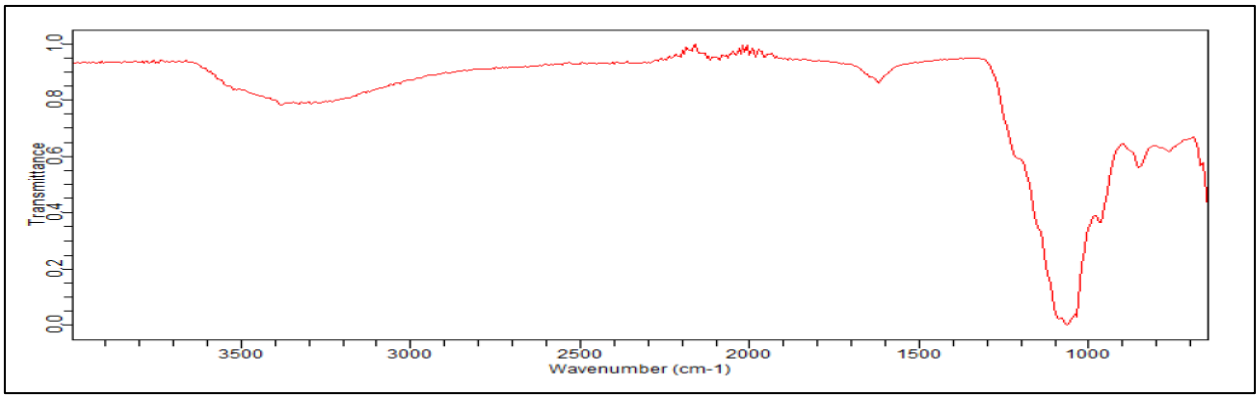

Figure 21 : Infrared spectrum of ZnONPs K

1.2. Biological activity tests of ZnONPs

1.2.1. Anti-inflammatory activity : Protein denaturation inhibition assay "BSA"

From the results shown in Figure 22. showed that AA-ZnONPs has more anti-inflammatory activity than the K-ZnONPs sample.

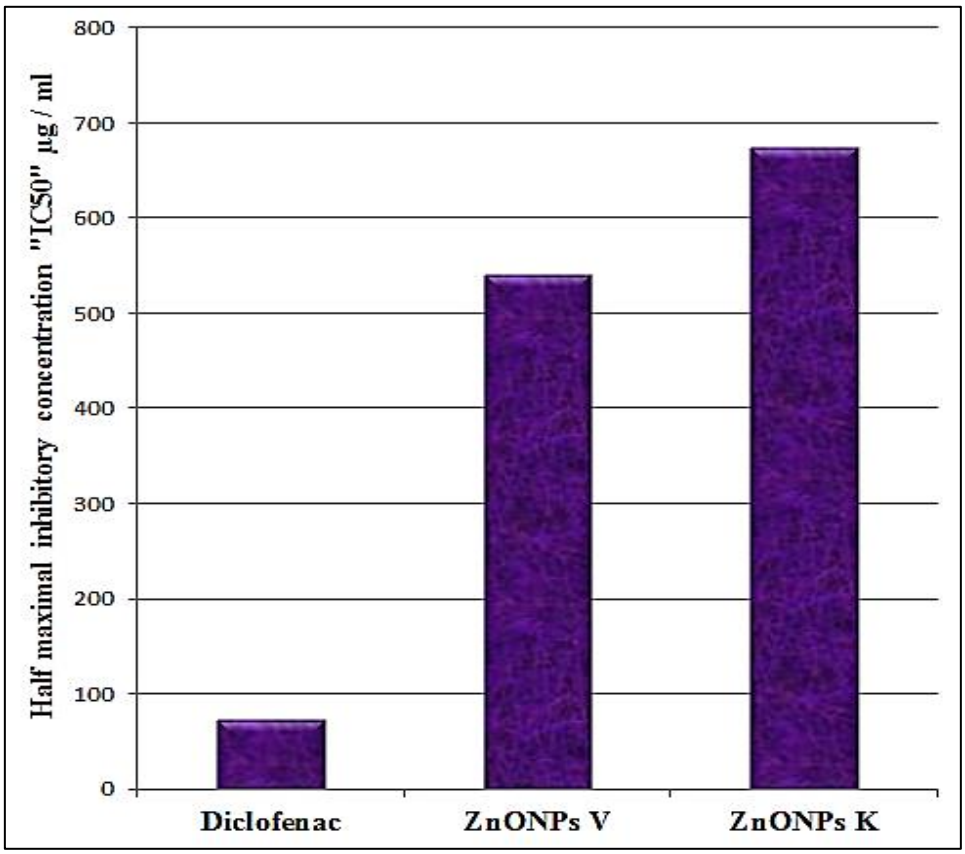

Figure 22 : IC 50 of Anti-inflammatory activity

1.2.2. Hemolysis assay

From the results shown in Figure 23. It showed that, ZnONPs syhthetized by ascorbic acid and KOH have an important anti-hemolysis activity with preference for the AS-ZnNPs than the K-ZnONPs.

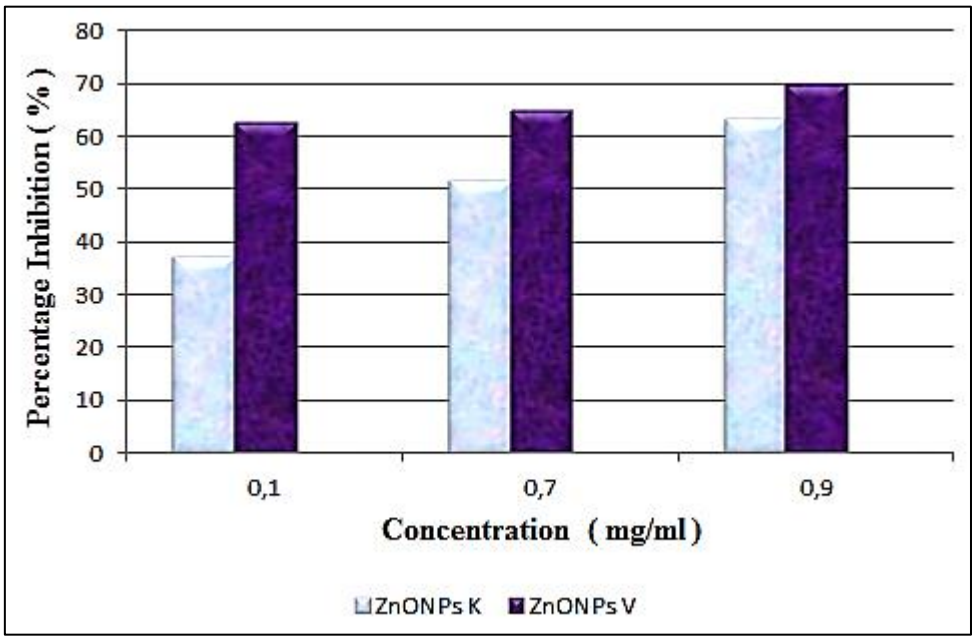

Figure 23 : Percentage inhibition of hemolysis Depending

on the concentration

2. Results of vivo study

2.1. Biochemical analysis

Regarding the biochemical parameters, we also performed kidney analyzes “urea crea” to support the results . And as expected, CPA group showed an increase in its levels compared to controls group “ $P < 0,05$ / $P < 0,001$ ”. Also , CPA increased the level of : sugar , TGP . The surprise was at the TGO level where there was no difference compared to controls group . As for testosterone, CPA caused a drop in its levels “ $P < 0,01$ ” . The three treatment regimens were able to modulation testosterone and blood sugar levels, including K-ZnONPs sample . The worrying thing is that all of the treatment regimens raised TGO levels , It also did not manage to reduce the levels of urea and crea , only the AA-ZnONPs sample managed to reduce urea . As for TGP levels, the surprise was that K-ZnONPs was able to reduce its levels, while the other samples raised its .

Table 04 : Biochemical markers of control and experimental group

	Controls	CPA	CPA + $Zn(NO_3)2$	CPA + AS-ZnONPs	CPA + K-ZnONPs
Urea (g/l)	0,3900±0,0753	0,467±0,04*	0,5050±0,1079	0,376± 0,083b	0,366±0,111
Creatinine (mg/l)	6,30±2,44	9,22± 0,53***	10,900±1,997*	9,420±0,847**	9,640± 1,019**
GOT (UI/l)	85,33± 5,86	93,33± 4,16	104,50± 4,04 **a	122,4±20,12*a	101,4±60,3*
GPT (UI/l)	25,00±2,65	38,50±4,36**	28,75± 7,14	39,60± 14,84	19,60± 11,19a
Blood Sugar (g/l)	1,50±0,087	1,95±0,57*	1,58±0,22a	1,64±0,23a	1,546±0,25a
Testosteroe (U/l)	1,020±0,19	0,22±0,14**	2,01±3,20a	0,908±1,36a	0,970±0,42a

Data are expressed as mean± SD (n= 5). * p<0.05, ** p<0.01, *** p<0.001: compared to controls group. a p<0.05, b p<0.01, c p<0.001 : compared to CPA group .

2.2. Hematological analysis

The results of Hematological analysis for all groups are shown in the table below : Where there is a significant difference in all markers between the control (group I) and CPA group (group M) (P < 0,05 / < 0,01 / < 0,001) . While AA-ZnONPs group corresponds with the control group in all markers except for only two (White blood cell and Lymphocytes) , and it differs with CPA group in all markers except one (Lymphocytes) . As for $Zn(NO_3)2$ group it differs in only two markers for the control group (Lymphocytes and Platelet) and three markers for the CPA group (Monocytes , Hemoglobin and Platelet) . K-ZnONPs group , which differs in three markers compared to the control group (White blood cell , Lymphocytes and Platelet) and four compared to CPA group (White blood cell , Monocytes , Granulocyte and Platelet) .

Table 05 : Hematological parameters of control and experimental groups

Markers	Controls	CPA	Cyclo + $Zn(NO_3)2$	Cyclo + AA-ZnONPs	Cyclo + K-ZnONPs
White blood cell(10^3/µl)	6,190± 0,520	5,238±0,542 *	5,108±0,771	3,326±0,908 **b	4,112±0,820 **a
Lymphocytes (10^3/µl)	3,007± 1,039	1,474±0,1851 ***	1,765± 0,359 **	1,462±0,361 **	1,770±0,445 **
Monocytes (10^3/µl)	0,473±0,186	0,680±0,0689 **	0,5200±0,0983 a	0,2800±0,1780 a	0,396± 0,105 b
Granulocyte (10^3/µl)	1,890±0,281	3,328±0,791 *	2,823±0,820	1,532±0,455 b	1,950±0,351 b
Red blood cell (10^6/µl)	7,123±0,309	6,422±0,285 **	6,932±0,391	7,222±0,283 b	6,532±0,604
Hemoglobin × 10^{-5} (g/µl)	12,567±0,833	11,575±0,340 I*	12,625±0,655 a	12,800± 0,474 b	12,000±1,061
Hematocrit (%)	38,57±2,20	34,980±1,472 **	38,33±2,43	39,700±1,007 c	35,90±3,12
Platelet (10^3/µl)	892,7± 25,4	1481,4±113,0 ***	830,5±35,4 *c	991± 241 a	1156,0±159,7 *a

Data are expressed as mean± SD (n= 5). * p<0.05, ** p<0.01, *** p<0.001: compared to controls group. a p<0.05, b p<0.01, c p<0.001 : compared to CPA group .

2.2. Oxidative stress markers

2.2.1. Malondialdehyde (MDA) levels

The results of MDA (Liver and Testicle) differed for the CPA group compared to the Controls (P < 0,05 / P < 0,001) , On the other hand, with regard to the liver MDA : The other groups are corresponds with the control group and differ with the cyclo group , Except for AA-ZnONPs group, which also differs with the controls . As for the testis results : All groups show a difference compared to the controls only .unlike AA-ZnONPs group, which shows the difference compared to CPA group.

Table 06 : MDA levels in Hepatic and testicular cells of control and experimental

Organs	Controls	CPA	CPA + $Zn(NO_3)2$	CPA + AA-ZnONPs	CPA + K-ZnONPs
Liver	8,390±1,661	12,52±6,45 *	9,06± 5,51 b	5,670±1,201 **c	6,609±1,942 b
Testicle	10,02±4,06	25,505±1,803 ***	34,75±9,83 *	18,90±6,44 a	33,57±7,58 **

Data are expressed as mean± SD (n= 5). * p<0.05, ** p<0.01, *** p<0.001: compared to controls group. a p<0.05, b p<0.01, c p<0.001 : compared to CPA group

2.2.2. Glutathione-S-Transferase (GST) activity

Regarding the liver, the results of CPA group show a difference compared to the control group (P < 0,05) . It is the same for K-ZnONPs group , in addition to the difference with the results of CPA group . While the other groups do not show any difference . This is different for the testicle: Where $Zn(NO_3)2$ and AA-ZnONPs group show a difference compared to CPA group (P < 0,05 / < 0,01) . and the other two groups : CPA and K-ZnONPs showed a difference compared to the controls (P < 0,05) .

Table 07 : GST activity in Hepatic and testicular cells of control and experimental groups

Organs	Controls	CPA	CPA + $Zn(NO_3)2$	CPA + AA-ZnONPs	CPA + K-ZnONPs
Liver	0,157±0,090	0,186±0,045 *	0,190±0,0327	0,187±0,0243	0,220±0,021 **a
Testicle	0,177±0,017	0,225±0,049 *	0,152±0,043 b	0,183±0,006 b	0,217±0,021 *

Data are expressed as mean± SD (n= 5). * p<0.05, ** p<0.01, *** p<0.001: compared to controls group. a p<0.05, b p<0.01, c p<0.001 : compared to CPA group

2.2.3. Superoxide dismutase (SOD) activity

Regarding the liver, the results of CPA group show a difference compared to the control group (P < 0,01) . It is the same for K-ZnONPs group (P < 0,05) . Also, ZnONPs V group shows a difference compared to the Cyclo group . As for the testicle results : The results of the CPA group differed from the control group (P < 0,05) , also $Zn(NO_3)2$ and AA-ZnONPs groups differed compared to the CPA group (P < 0,05) .

Table 08 : Hepatic and testicular SOD levels of all groups of rats in addition to P value

Organs	Controls	CPA	CPA + Zinc S	CPA + ZnONPs V	CPA + ZnONPs K
Liver	11,64±4,31	6,42±3,05 **	9,27±4,67	11,59±4,92 b	4,08±3,84 *
Testicle	12,436±1,186	11,339±1,202 *	13,080±1,817 a	13,879±1,716 a	11,90±2,47

Data are expressed as mean± SD (n= 5). * p<0.05, ** p<0.01, *** p<0.001: compared to controls group. a p<0.05, b p<0.01, c p<0.001 : compared to CPA group

2.3. Histological results

2.3.1. Hepatic tissue

Hepatic histological analyzes the control group showed a very healthy structure free from various deformities . In contrast to the hepatic sections of CPA group, which showed several negative effects like : cell lysis , hemorrhage and necrosis . While these effects partially disappeared in the rest of the groups. Only AA-ZnONPs group showed very slight hemorrhage , And to a great degree. in K-ZnONPs group . With figures key : Triangle ; Normal cellular tissue ,Circle ; hemorrhage , Square ; necrosis , star ; cell lysis.

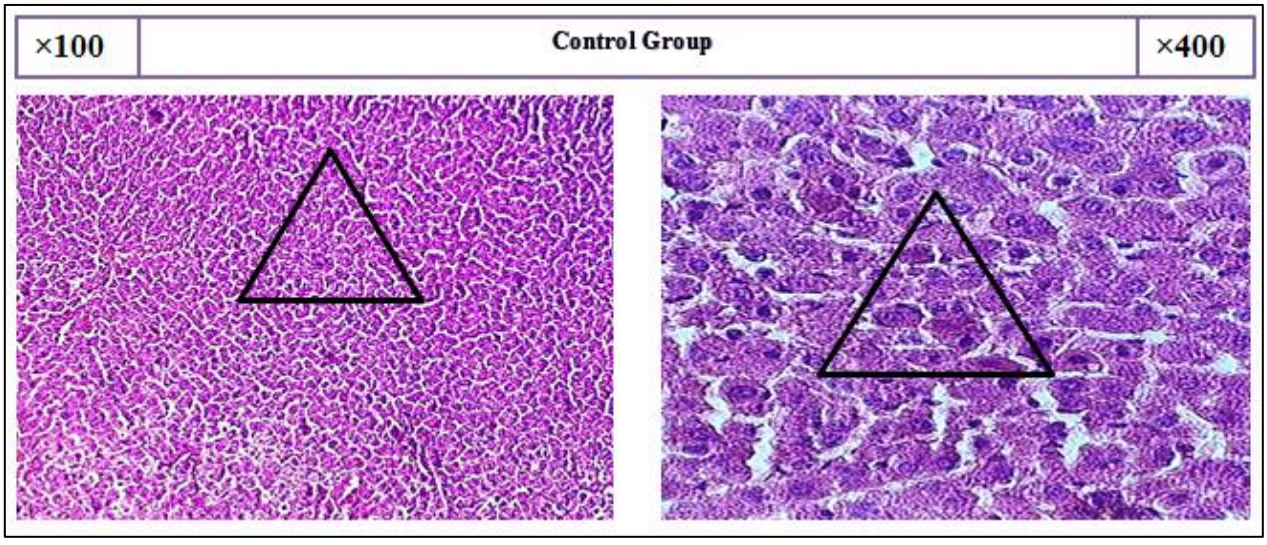

Figure 25 : Hepatic histological sections of controls group

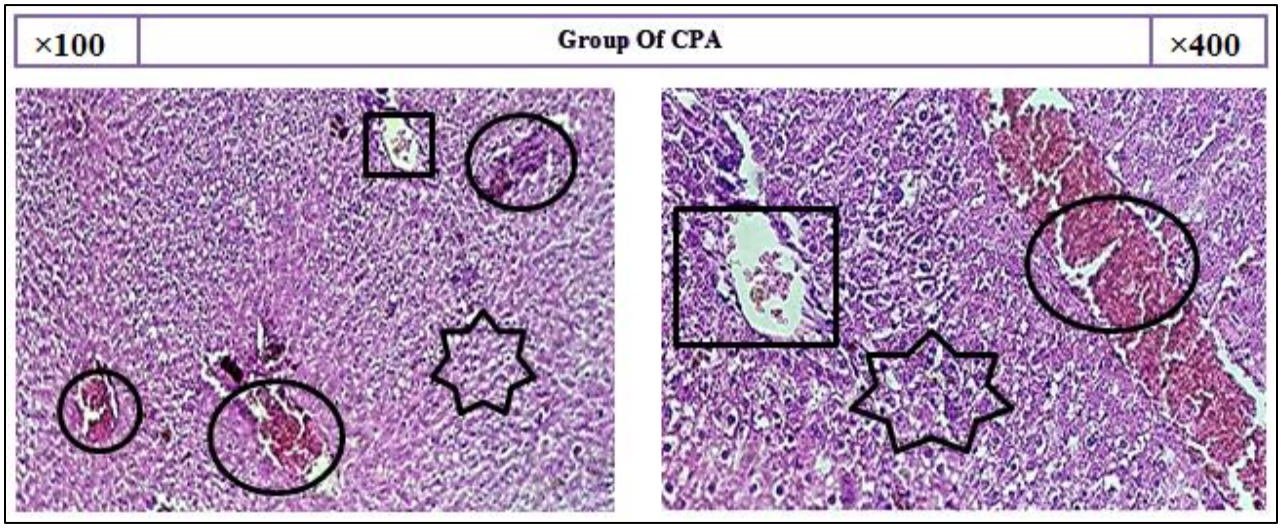

Figure 26 : Hepatic histological sections of CPA group

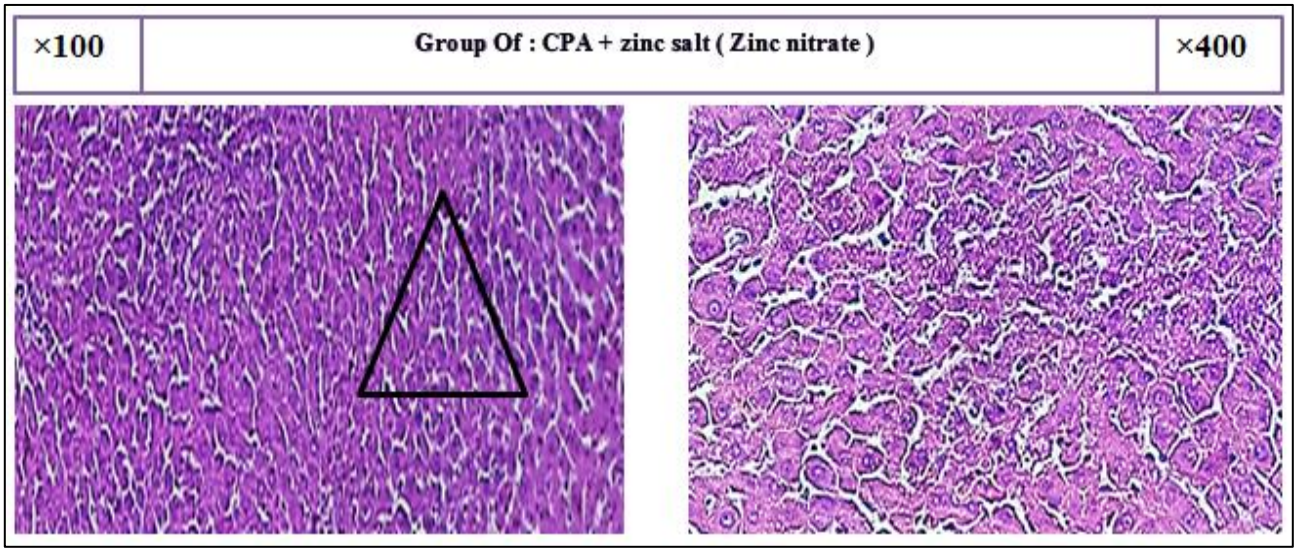

Figure 27 : Hepatic histological sections of Zn S group

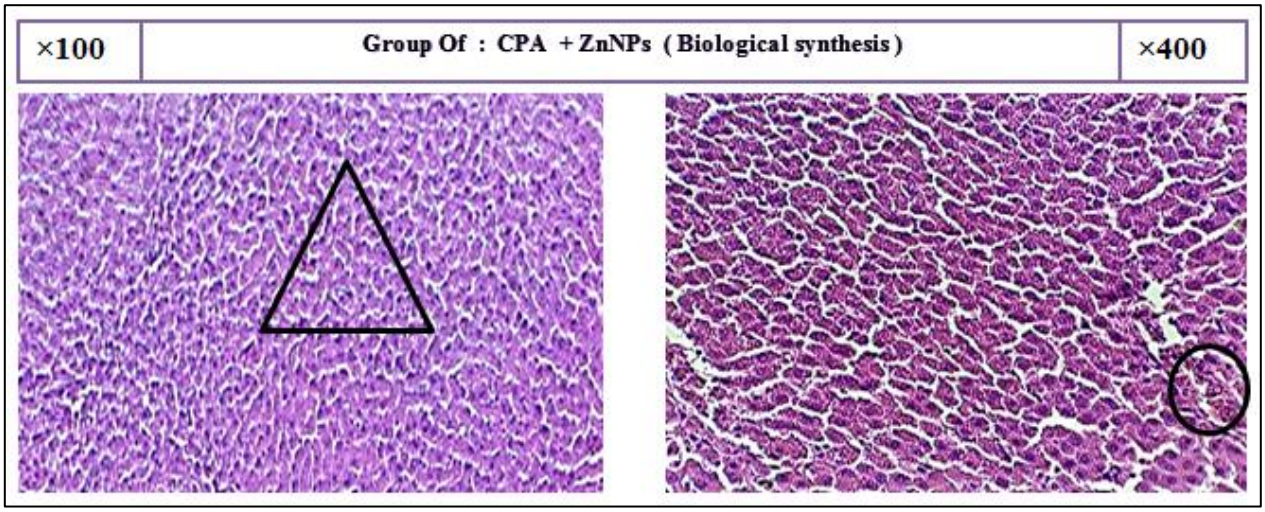

Figure 28 : Hepatic histological sections of Zn ONPs V group

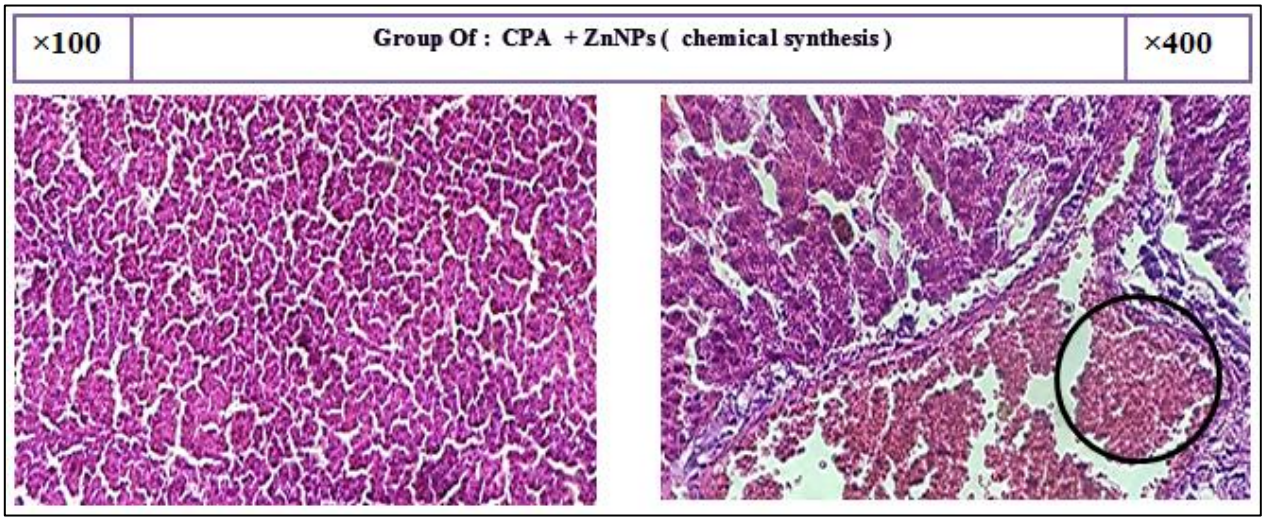

Figure 29 : Hepatic histological sections of ZnOnPs K group

2.3.2. Testicular tissue

Microscopic images of the testicular tissue sections of the controls group evealed a healthy structure of the testicle, in which the seminiferous tubule appear in their normal form (some seminiferous tubule were elongated as a result of our routine work on the testicles) , The various stages of spermatogenesis appear well . In contrast to the tissue sections of CPA group , Where destroyed and decomposing seminiferous tubule appear, other tubes may also appear in which spermatogenesis may stop . These features partially disappeared in AA-ZnONPs group . And almost similar to the natural sections. Followed by the histological sections of Zinc S group least similar. In contrast to the tissue sections of K-ZnONPs , which resembled the sections of the CPA group, and similar disorders appeared . With figures key : oval circle ; Lumen seminiferous tubule , Square ; interstitial cells , yellow arrow ; Normal seminiferous tubule , black arrow ; Spermatids , brown arrow ; Basal lamina , green arrow ; Spermatozoa , red arrow ; Spermatogonia , star ; destroyed seminiferous tubule , triangle ; Residues of destroyed seminiferous tubule.

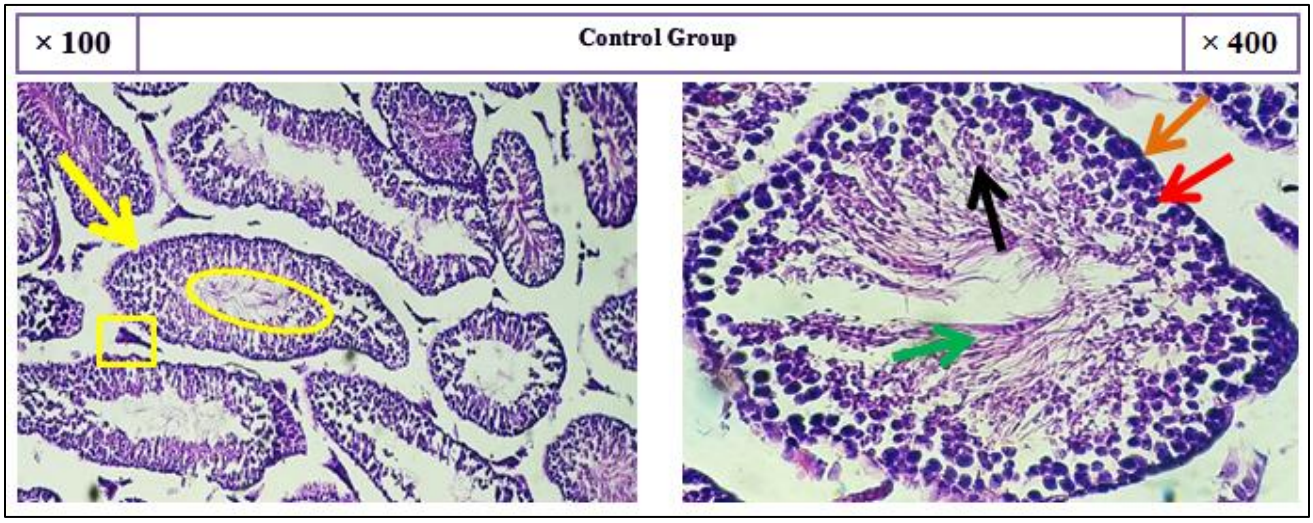

Figure 30 : Testicular histological sections of control group

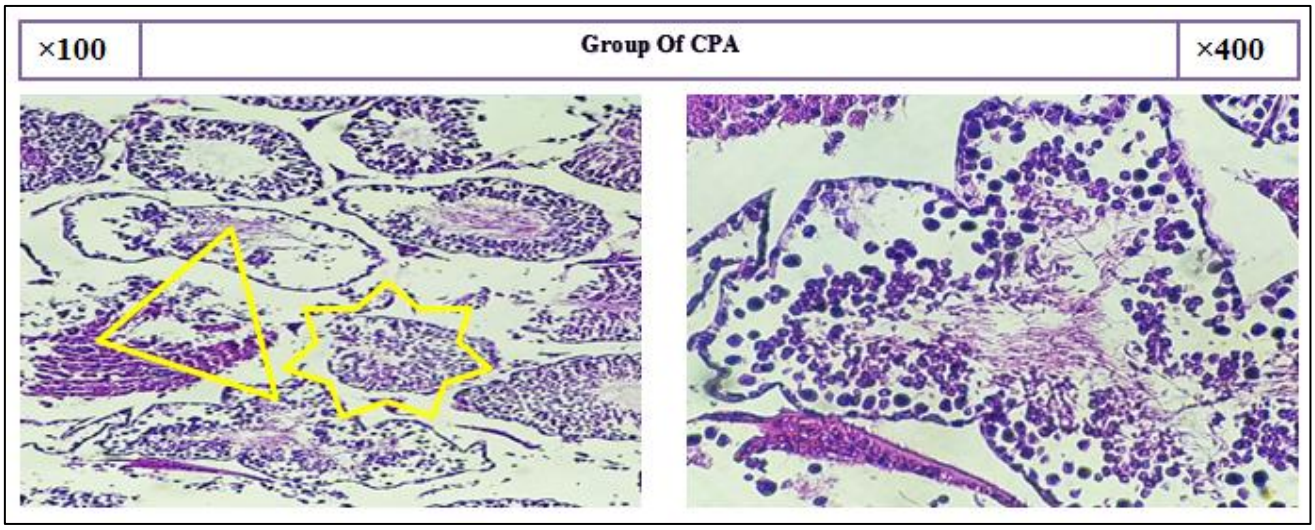

Figure 31 : Testicular histological sections of CPA group

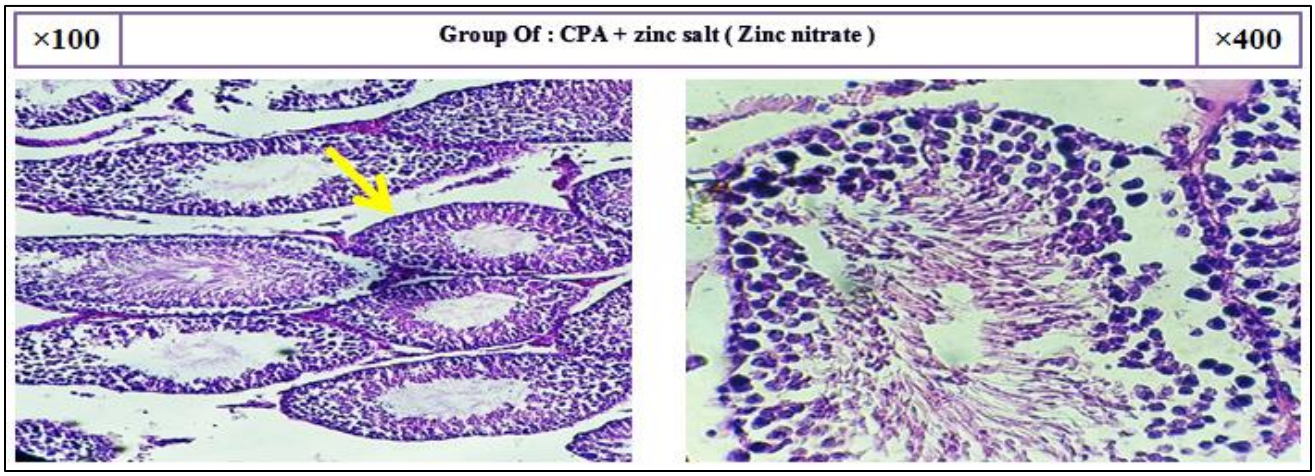

Figure 32 : Testicular histological sections of Zn S group

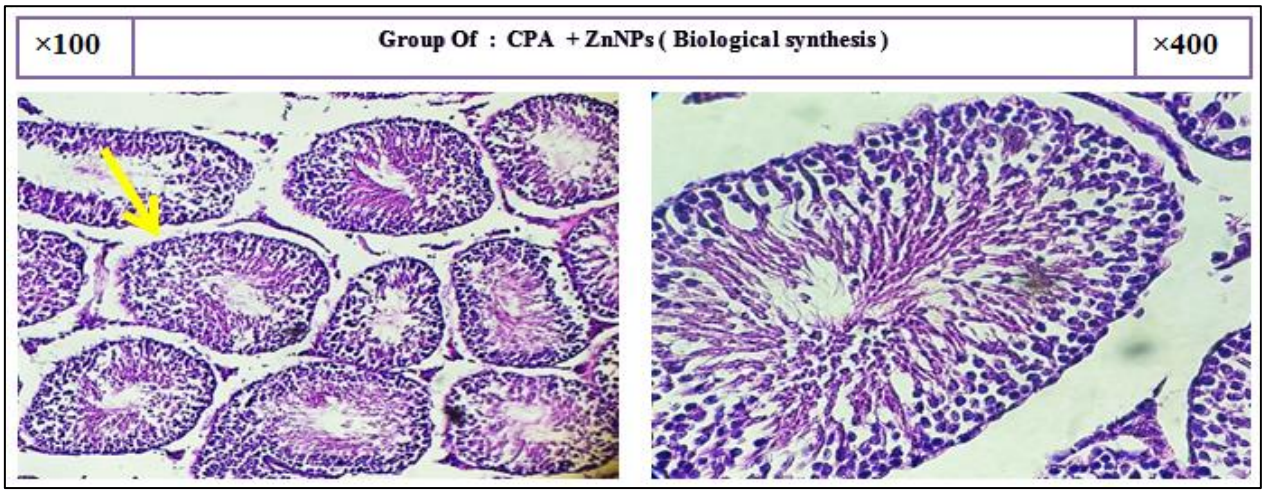

Figure 33 : Testicular histological sections of ZnONPs V group

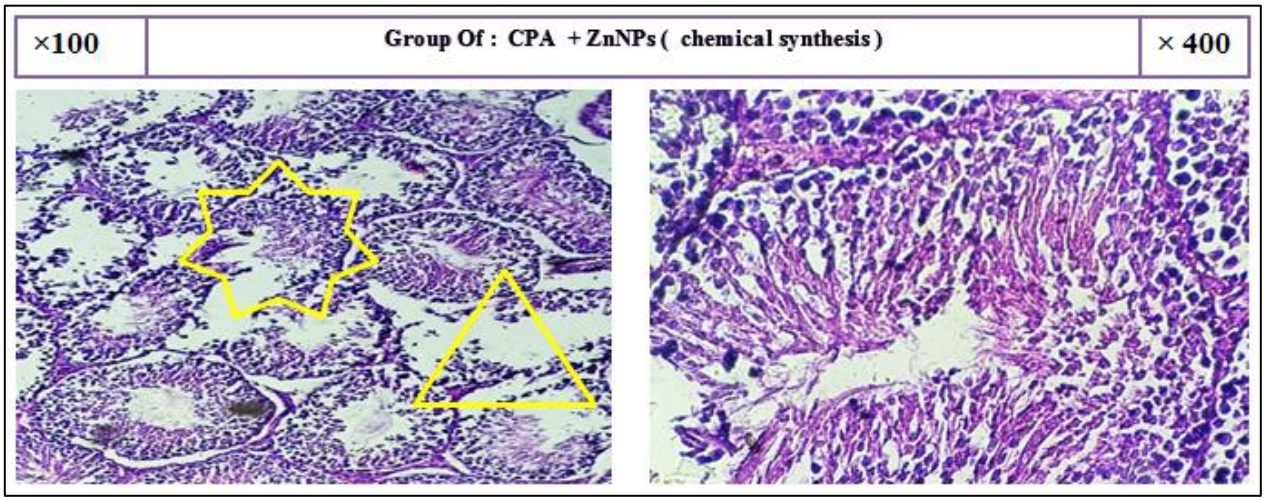

Figure 34 : Testicular histological sections of ZnONPs K group

Chapter III:

Discussion

Discussion the results of vivo study

Cyclophosphamide (CPA) is an anti-cancer chemotherapeutic drug , It is characterized by alkylation Property (Nandini Bhat and al.,2018) . It intervenes directly at the DNA and prevents the division of cancer cells (Alsadiq Al Hillan and al.,2019) . It is used alone or in combination with other drugs (Khalid M. .Maya and al.,1989) . CPA is inactive, and it is activated in the liver by CYP450 , to product 4-HO-CP which give aldophosphamide , Then give the key metabolites with a non-retroactive reaction (James L and al.,2000) . One of these metabolites is: phosphoramide mustard , It is the metabolite responsible for the bifunctional alkylating (Lois J.Ayash and al.,1992) . The other is Acrolein metabolite Which is associated with oxidative stress and negative effect of CPA (Kartick Patra and al.,2012) . CPA targets cells that are highly dividing, which is the feature of cancer cells, but it is feature of some other healthy cells in the body as well, and this is the reason for the toxic effects of CPA (CA Slater and al.,1999) . Male germ cells are characterized by high frequent division, until sperm are formed. This exposes the testis to the effects of CPA (Juárez-Rojas and al.,2017) . On the other hand, the liver is responsible for purifying and detoxification of the body, but it is at risk through its excessive exposure to drug , for example CPA (Ryan J. Schulze and al.,2019) . Where , A study in 2017 demonstrated the toxicity of CPA on the liver, even with the low doses (Chakkor A and al.,2017)

1.Biochemical Markers

The importance of hepatic functions : responsible for maintenance of normal homeostasis and physiological functions , for the body reinforces the necessity for the organ to remain healthy (ROBERT R. MARONPOT and al.,2010) . Evaluation of hepatic enzymes is very important for diagnosing the state of the liver (Philip Hall and al.,2012) . CPA is metabolized in the liver, which exposes it to its effects more, and despite the negative effects of CPA on the liver - which we will explain later - only a few studies have proven an increase in liver enzymes in the blood after CPA administration (Zainab NH Anber , 2018) . No less important is kidney function also, and among the criteria that are used to test the health of the kidneys are Urea and Creatinine (Chris Isles and al.,1996) . Hepatic enzymes like (AST or GOT, ALT or GPT, ALP and LDH) normally is located in inside of hepatocyte , and because CPA causes liver tissue injury, it is expected that these enzymes will leak into the blood (Mahsa Zarei and al.,2013) .Is the same for the kidneys , Where CPA causes kidney tissue injury and urea crea leak into the plasma (Sabry A. El-Naggar and al.2015) .As for our results , CPA group showed a high significant difference in

GPT levels and non-significant difference at GOT levels , and the reason for this could be the following : ALT is primarily localized to the liver and is considered a more specific test for liver damage (ODIBA, Arome Solomon and al.,2014) . The values of urea crea showed a clear difference compared to the controls group .

Treatment regimens " $Zn(NO_3)2$ and AA-ZnONPs " were unable to lower levels of GPT /GOT, only ZnONPs K sample was able to lower GPT levels . Although a 2012 study revealed that giving orally zinc "50mg/Kg" with vitamin C "100mg/Kg" modifies - lowers - the activity of liver enzymes (E.D. Eze and al.,2012) . Another study proved that zinc deficiency increases the activity of this enzymes (M.I. Yousef and al.,2002) . Despite that, no improvement was shown, especially on the water values, GOT increased more.

On the blood sugar level , CPA group showed a significant difference in the results compared to the controls group . This is due to : CPA is able to reduce the levels of hexokinase and glucose 6 phosphate dehydrogenase which results hyperglycemia (M.NAJMA HABEEB and al.,2013367). All treatment regimens were able to lower blood sugar , and all groups showed an equal difference compared to CPA group . This is due to : ZnONPs lead to increased liver glucose uptake and storage, and inhibition of intestinal α-glucosidase (Samy Ali Hussein and al.,2014365) . Also , In skeletal muscles and adipose tissue Zinc-α2-glycoprotein increases cellular GLUT4 protein which resultant increase in glucose uptake (Priyanga Ranasinghe and al.,2015366) . Which leads to low blood sugar . The increase in blood glucose, transaminases activities and urea level can be explained by the effect of insulin secretion activity as a result of the effect of cyclophosphamide on the one hand, and also on the other hand it is explained by the accumulation of amino acids such as alanine and glutamic acid in the blood resulting from the breakdown of proteins in the body. Thus, under the action of transaminases, these amino acids can be converted into carboxylic compounds like keto-glutamic acid and pyruvic acid, and then into glucose, resulting in metabolic residues like urea; this demonstrates transaminases' high enzymatic activity (Derouiche Samir and al.,2016) .

Zinc has a critical role in the synthesis, storage, and secretion of insulin, as well as the structural integrity of insulin in its hexametric form. When zinc levels are low, the islet cells' capacity to manufacture and secrete insulin is harmed (Maret W and al.,2017). The protein tyrosine phosphatase1B (PTP 1B), a critical regulator of insulin receptor phosphorylation state, is known to be a zinc-ion activation target. Zinc has been found in studies to help improve

peripheral insulin sensitivity by potentiating insulin, which promotes glucose transport (Samir Derouiche and al.,2017) . which can be ameliorate the blood glucose level in group treated with zinc. Zinc is required for the activation of several other enzymes, including DNA polymerase, RNA polymerase, alkaline phosphatase, and superoxide dismutase (SOD). As a result, researchers have hypothesized that zinc supplementation could improve liver function and reduce cancer risk (Hosui A and al.,2018). Caspases are a family of cysteine proteases that promote apoptosis, or the planned death of cells. Zinc is an essential regulator of caspases. Zinc has an effect on the activation and inhibition of a number of molecular processes. Zinc can imitate the effects of hormones, growth factors, and cytokines as a component of intracellular signal molecules. The stability of free intracellular Zn++ ions has a significant impact on the onset and progression of a variety of illnesses. Numerous proteins are involved in maintaining intracellular zinc homeostasis. The existence of such regulations emphasizes zinc's critical role (Grüngreiff and al.,2016) . Therefore, the supplement in zinc made it possible to increase the stability of intacellular zinc level, which enabled a decrease in the activity of transaminase and urea level in the blood.

As for testosterone , testosterone is a steroid sex hormone is often considered and called male sex hormone and metabolized from cholesterol by desmolase activity (Jaroslava Durdiakova and al.,2011) . Testosterone is synthesized at the level of the leydig cells in the testicle , and it is necessary for spermatogenesis , and its disorder can lead to male infertility (Aditi Sharma and al.,2020) . 17ß-HSD and 3ß-HSD they are essential enzymes in the biosynthesis of steroid hormones in leydig cells , and any defect in these enzymes affects the production of testosterone (Małgorzata Kotula-Balak and al.,2012) . It has been shown that CPA is involved in the decrease in the activity of these enzymes, which is reflected in the levels of testosterone (Azubuike Peter Ebokaiwe and al.,2021) .

This is also evident in our results, as the CPA group showed a high significant difference compared to the control group . And all three treatment regimens were able to restore testosterone levels to their levels, and showed almost equal significant difference compared to the CPA group . There is a direct action of zinc deficiency on testicular steroidogenesis (S. A. HAMDI and al.,1997) .

Through a 2008 study, it was proven that zinc raises the levels of 17ß-HSD and 3ß-HSD (Nermin A. H. Sadik,2008) . Another study in 2020 also proved that ZnONPs raises the levels of 17ß-

HSD and 3ß-HSD(Huijuan Kuang and al.,2020) . On the other hand, regarding the risk of CPA lowering testosterone levels .

2.Hematological Markers

In addition to using CPA as an anticancer chemotherapy, it is also used to suppress bone marrow (Huimin Yan and al.,2021) . The reasons for the suppression are due to the alkylation property of CPA. , and depending on the dose, it also affects the healthy cells that are dividing rapidly , as it leads to the alkylating of the DNA and causes apoptosis of haematopoietic cells (Samah AboZaid and al.,2020) . Which can develop into all types of blood cells (Ji Yoon Lee and al.,2019294) . CPA depends on bax and caspase-3 to activate apoptosis (Jiahong Han and al.,2018) . CPA metabolites can also interact with erythrocyte membrane proteins and destroy them (NWANKPA Promise and al.,2014) . Which of course leads to lower levels of : RBC ,Hb and Hematocrit .

This explains our results , where all the immune cells and Red blood cell of the CPA group showed a clear difference compared to the control group , This matches the results of a study in 2017 , in which the effects of a plant extract against these negative effects of cyclo were evaluated (Sawsan Ahmed Abd Elhalim and al.,2017) And by another study in 2020 (Farhana Fatema and al.,2020) . Lymphocytes were greatly suppressed, Because it shows a a low ALDH enzyme levels. , this puts the cells at greater risk for the effects of CPA (Amy E. DeZern, MD and al.,2011) .

In contrast to the levels of platelets that are supposed to decrease as well, CPA group showed very high values of platelets compared to the control group and we can explain this , a study in 2019, demonstrated that 150 mg / Kg of CPA was only able to suppress 54% of platelet count and 92% of leukocyte count (Adnan AYHANCI and al.,2019) . This, of course, explains how our dose “90 mg / Kg”did not suppress platelets. and it can be raised as a result of inflammation, anemia, or as a drug reaction (Quyen T. Vo and al.,2018) .

Zinc is an essential mineral in the human body, and immunity is one of the main directions of zinc (Richard J. Wood,2000300) . But during exposure to high concentrations can cause suppression of the immune system (Inga Wessels and al.,2017301) This could explain the absence of any improvement in the results of the $Zn(NO_3)2$ and K-ZnONPs group , as for the AA-ZnONPs group , It seems that the reduction of ZnNPs using vitamin C had a more

immunosuppressive effect as the average white blood cell count was 3, this could be due to the very effective absorption of AA by white blood cells is through SVCT proteins " 50- to 100-fold higher than plasma concentrations " (Giuseppe Cerullo and al.,2020) . where a high doses can act as a pro-oxidant than an antioxidant (Martin Doseděl and al.,2021) ,this is what damages the immune cells . This is similar to the results of a recent study conducted in 2020 , where the use of ZnONPs against CPA increased the suppression of white blood cells (Karema El M Shkal and al.,2020) .

RBC are the most abundant cells in the body. They contain the protein hemoglobin, which is mainly responsible for transporting O2 from the lungs to the rest of the body. It also fixes CO2 to expel it from the body (Zuhairah Ismail Muhammd and al.,2022) . Which means that low levels of RBC can lead to disaster in the body: hypoxia, cardiovascular disease (Viktoria Kuhn and al.,2016) . This applies to the CPA group, which showed low levels of erythrocytes and hemoglobin . We previously explained that low levels of RBC in this group results from the apoptosis of haematopoietic cells . where CPA depends mainly on Bax and caspase-3 . In our study , and although it has been proven that zinc has the ability to inhibit caspase-3 (David K. Perry and al.,1997) , however, zinc salts did not show an effect against CPA . Where the levels of RBC for Zinc S group did not show any improvement . Contrary to these results, the zinc nanoparticles reduced with ascorbic acid showed that the values of RBC returned to the normal level , we explain this by reaching an appropriate amount of zinc for red blood cells, as zinc enables the bone marrow to form new red blood cells even after exposure to anemia (Yen-Hua Chen and al.,2020) . This is enhanced by ascorbic acid, which is involved in several antioxidant mechanisms (Viktoria Kuhn andal.,2016) that can suppress OS resulting from the interaction of CPA-metabolites with the membrane of RBC . All these positive effects of ZnNPs vanished when they were reduced with chemicals agent . where the levels of RBC did not show any improvement for K-ZnONPs group .

3.Oxidative stress

Although CPA is used mainly to inhibit cancer cells and suppress immunity, it is associated with many toxic effects in the body on various organs (Barbara F. Hales ,1982) . Several studies have suggested that oxidative stress is responsible for toxicity, including liver and testicular toxicity, since CPA is closely related to stress (Mohammad Shokrzadeh and al.,2014). Oxidative stress results from an imbalance of oxidants with antioxidants (B. Halliwell , 2007).

which can lead to many negative effects including : lipid peroxidation , mutations of DNA , depolymerisation of polysaccharides , protein oxidation (H. Cottier and al.,1995) .

As evidence of this, a very important study was conducted in 2020, which demonstrated that injecting mice with CPA “20mg/Kg/day” and ZnONPs “5mg/Kg/day” for 14 days together reduced the oxidative stress caused by CPA and increased levels of antioxidants (Karema El M Shkal and al.,2020)

Recent studies have demonstrated that ZnONPs have different biological activities, the most important of which is anti-oxidation against oxidative stress (Ture Safawo and al.,2018) .but green synthesis has better antioxidant properties than chemical synthesis, This is due to the reduction and stability and capping of nanoparticles by biological compounds (A. Muthuvel and al.,2020) . Green synthesis of ZnONPs is done by adding the plant extract, but it turns out that the plant extract has less antioxidant activity than ZnONPs product from it , which in turn is lower than ascorbic acid (S. Rajeshkumar and al .,2018) . to achieve both, ascorbic acid can be used directly to synthesis ZnONPs (Natpasit Chaithanatkun and al.,2015) .

One of the organs affected by oxidative stress of CPA is the liver , which occupies an area and great importance in the human body ,where it is responsible for metabolism : of carbohydrate , Cholesterol , Lipid and Drug detoxification (Zaenah Zuhair Alamri ,2018) . Which means that its toxicity leads to disaster in the body (Richa Sachan and al.,2018) .on the other hand the testicle as well , which is the male reproductive organ that contains the seminiferous tubules , where spermatozoids are produced (CARL G. HELLER and al.,2016) . and its toxicity leads to poor spermatozoids production, which means poor fertility (Toshinobu Miyamoto and al.,2012)

In this study, oxidative stress of hepatic and testicular tissue was evaluated by determining the values of : MDA “ a product of Lipid peroxidation (Edwin Ho and al.,2013) ” , SOD “An enzymatic antioxidant involved against oxidants in the body (Yuji Naito and al.,2010)” . GST “Participates in protection against oxidative damage (J Bhagat and al.,2016)” .

ACRO or 2-propen-1-a “metabolite of CPA”, is a reactive ubiquitous α, β-unsaturated aldehyde highly reactive such as free radicals and more it causes lipid peroxidation of polyunsaturated fatty acids (Wang-Sen Qin and al.,2014) . by activating of iNOS “isoforms of NOS : nitric oxide synthase (Qingjie Xue and al.,2018)” The formation of NO leads to the production of peroxynitrite , which leads to LPO and cellular energy crisis (A. Korkmaz and

al.,2007) . on the one hand , ACRO can react with xanthine oxidase to be converted back into superoxide , which in turn leads to the lipid peroxidation (JAMES D and al.,1993) . the danger of ACRO is that it has a half-life several times longer than free radicals, and is also considered one of the products of LPO (R. Shi and al.,2015) . Which leads to a closed loop of oxidative stress , being a product and at the same time a catalyst for LPO .

The hydroperoxides resulting from the LPO undergo fragmentation to produce a three-carbon compound : malondialdehyde “MDA” Able to interact with other compounds (Mariona Jové and al.,2020) .

CPA metabolites cause repression of gene expression of Nrf2 molecules “Nuclear factor erythroid-2 related factor 2” (Prashant R. Gore and al.,2016) . which is a transcription factor in expression of a series of antioxidant genes including : genes of SOD (Bin Ni and al.,2021) . This means that CPA aims to reduce SOD levels in an indirect way.

Also , CPA causes the generation of free radicals such as : superoxide radicals (Elangovan Selvakumar and al.,2005) . SOD is responsible for dismutation of superoxide $O2^-$, which leads to lower SOD levels in case of increased $O2^-$ production (Filip Cristiana and al.,2014) .

GST are a super family of enzymes Involved in phase II detoxification reactions , GST safeguard the cells against chemical-induced toxicity by catalyzing S-conjugation between the thiol group of GSH and electrophilic moiety in the hydrophobic and toxic substrate (Smita Kumar and al.,2018) . During the occurrence of disturbances in the body, such as exposure to chemotherapy, for example, an increase in the activity of GST occurs and a decrease in the levels of GSH (Edita Baltruskeviciene and al.,2016) . GST is essential in removing CPA toxicity from the body , by addition of nucleophiles compound such as glutathione to the site of unsaturated ACRO , This reduces the reaction and toxicity of ACRO (Daniel J. Conklin and al.,2015) . CPA can also increase GST levels by inducing oxidative stress, as GST metabolizes products of oxidative stress (Dasari S and al.,2018) .

3.1.At the hepatic level

ALDH is essential enzyme in neutralizing the toxicity of ACRO and converting it to inactive or less toxic metabolites, hepatocytes in particular produce high levels of ALDH and this is what protects them slightly from CPA toxicity (Idriss H. Mohamed .,2021291) . For this reason, we obtained results of low oxidative stress markers in the liver compared to the testis.

Our study showed a significant difference between the results of the control group and the CPA group , This is due to the CPA mechanism in generating of MDA . This is what was shown by a very recent study in 2021 , only rats were injected with a higher dose compared to the dose in our study : 40 mg / kg for five consecutive days (Zhiying Zhang and al.,2021) . and shown by a study in 2006 , where the injection of rats with a dose of CPA "75 mg / kg for one day only" less than our studied dose "30 mg / kg for three days", caused an increase in MDA levels (Arvind Lal Bhatia and al.,2006) .

As expected also for the results of SOD , hepatic SOD levels showed a significantly higher decrease for the CPA group compared to the control group , this is due to the CPA mechanism in reducing the SOD . This was proven by a study in 2009 , in which injecting mice with a dose lower than our studied dose of 75 mg / Kg , caused an increase in MDA levels and reduced levels of the most hepatic powerful antioxidant : GSH and SOD (D.N. Tripathi and al.,2009286) .

Low GSH levels reflect GST levels in our study , where we previously explained that GST is responsible for ridding the body from CPA toxicity, and GSH is needed as a substrate for this . This means a lower level of GSH and a higher activity of GST .

The treatment regimens : Zinc S and ZnONPs K were not able to reduce hepatic oxidative stress by a significant percentage only, which reduced the levels of MDA compared to CPA group . This means that zinc salts were able to reduce MDA levels, and this was proven by a study in 2003 (AHMET OZTURK and al.,2003) . By decreasing LPO (Yuvaranjani Gali and al.,2019) . As we previously indicated , ZnONPs has a antioxidant activity and green synthesis has more antioxidant activity than chemical synthesis , And this is reflected in the results of MDA levels . when ZnONPs K group , showed a high significant difference compared to CPA group . while the best results were for AA-ZnONPs group , which showed a very high significant difference compared to CPA group , and a high significant difference compared to control group

, this means that the MDA levels are lower than the control group as well . In general and at the hepatic level in particular , ZnONPs can prevent LPO " Prevent damage to the hepatocyte membrane " by reducing the levels of free radicals and thus decreasing MDA production (Samir A.E. Bashandy and al.,2017) . and the reduction of ZnNPs by ascorbic acid could be the reason for the significantly reduced levels of MDA , as mentioned earlier, high levels of NO can cause LPO , while AA can control NO levels, prevent LPO and reduce MDA levels further in hepatic tissue (Tanmoy Rana and al.,2010) .

Zinc has the ability to modulate Nrf2 activity (Krishna Prahlad Maremanda and al.,2014) . However, $Zn(NO_3)2$ and K-ZnONPs groups did not show any improvement in the SOD results . While ZnONps V sample was able for returning SOD its normal level . This is due to a sufficient amount of zinc reaching the liver cells to modulate the activity of Nrf2 . This is enhanced by the antioxidant activity of ascorbic acid, which inhibits free radicals and modulates Nrf2 activity as well (Aaron J and al.,2016) . Also, a recent study in 2018, proved that combining it with vitamin E also leads to Nef2 activation (Radhakrishnan Chandraprabha Vineetha and al.,2018) .

3.2.At the testicular level

Testicular tissue is particularly vulnerable to the effects of CPA , as it is rapidly dividing as cancer cells (Hoda H. Anan and al.,2017) . This explains the higher levels of oxidative stress in the testicles compared to the liver . Oxidative stress causes severe damage to the testicles and leads to infertility, as it affects : motility and count of sperm , sperm-egg recognition, and sperm-Oocyte fusion (R John Aitken , 2020) .

Regarding the MDA levels in the testicular tissue , the results showed a very high significant difference between the results of the control group and the CPA group " very high MDA levels ", this is due to the same mechanism of MDA production explained previously . Where this was clearly proven through a study on mice in 2020 , as the testicle is exposed to the effects of the CPA to a large extent, it shows signs of oxidative stress, including elevated MDA (Maha I. Alkhalaf and al.,2020) . As for SOD results , CPA group showed a significant difference compared to controls group , through the same mechanism proposed at the hepatic level, SOD were reduced by CPA . And this was proven by a study in 2005, where administering a dose of

150 mg / Kg orally of CPA led to a decrease testicular SOD levels (Elangovan Selvakumar and al.,2005[316]) .

In contrast to the liver, the treatment regimens " $Zn(NO_3)2$ and K- ZnONPs groups " were unable to lower MDA levels and did not show a significant difference compared to the CPA group . While AA-ZnONPs sample was able to reduce MDA levels , This is due to the same reasons mentioned above , and to the fact that ascorbic acid is also able to react with O2- , "which leads to the LPO" in the extracellular fluid thus protecting sperm viability and motility (Ashok Agarwal and al.,2014) .

On the other hand, we mentioned the effective role of zinc in modulating the activity of Nrf2 , which in turn is responsible for the gene expression of antioxidants such SOD . And this could be the reason behind the high levels of SOD when treating with $Zn(NO_3)2$ and AA-ZnONPs compared to CPA group . All antioxidant enzymes require a cofactor to maintain their catalytic activity. Thus, mitochondrial SOD needs manganese but cytosolic SOD requires copper and zinc for their activity. The mechanism of what could be the ZnNPs enter in the target cells where it dissociates and slowly but consistently released as Zn+2 ions .

It is well known that Zn is a powerful antioxidant metal; it is the core constituent of antioxidant enzymes such as SOD and a recognized protector of sulfhydryl groups . The antioxidant activity, which then detoxify the free radicals. These factors protect cells from ROS damaging (Siddiqi KS and al.,2018, Kurutas EB,2016) .

We previously discussed the stages of CPA metabolism , just as GST plays a role in ridding the body of CPA toxicity . Also ADH it converts the active CPA metabolite to an inactive metabolite and thus eliminates the CPA activity before the need for GST . This means that the high activity of ADH enzyme reduces the activity of the GST . ADH is a zinc-dependent enzyme (Václav Tvrdý and al.,2021) . This means, it was expected that zinc contribute to reducing the activity of GST . Although this has not been demonstrated in the liver, it has been well demonstrated in the testis , where The two samples "$Zn(NO_3)2$ and AA-ZNONPs " were able to reduce GST levels .

4.Histological Sections

Organs toxicity is a very sensitive limit to the dose of CPA , where ACRO causes oxidative tissue injury , through produces highly reactive oxygen free radicals (K.B.H. Kumar and al.,2005318, Joseph Gbenga Omole and al.,2018) . and altering the levels of MDA , GSH , SOD, GPx , GR , CAT in favor of oxidative stress (Sadashivaiah Jnaneshwari and al.,2013) . CPA is implicated in : hemorrhage of various organs after bone marrow destruction (Seymour Levine and al.,1973) , responsible for inducing apoptosis through increase expression of the pro-apoptotic molecule BAX (Archana Adhikari and al.,2021) . Hepatic and testicular tissues are among the most important tissues exposed to CPA toxicity (Azza A. El-Sheikh and al.,2017) .

4.1.Hepatic Sections

The liver is associated with many vital and central functions " metabolism of : Vitamin and Mineral , Protein , Carbohydrate , lipide . Lipogenesis " in the body, which reflects the risk of damage to the hepatic tissue (Faegheh Zaefarian and al.,2019) . CPA leads to impaired functional status of the liver resulting to elevated serum ALT , AST , tissue MDA, and reduction in anti-ox markers . generally , drug mediated liver toxicities are characterized by cell necrosis and injuries elicited by inflammation (Azubuike Peter Ebokaiwe and al.,2021) .

CPA is implicated in increased levels hepatic enzymes in plasma GGT / ALP as result of impairment of intrahepatic and extra-hepatic bile flow " cholestasis " (Ebenezer Tunde Olayinka and al.,2015326) , acute cholestasis is capable of causing necrosis . Necrosis shows disturbance of membrane integrity and disintegration of organelles, and cell lysis (Kewei Wang , 2014327) . It was also observed by Hutheyfa Abdulhussein Al-Salih and his colleagues Through a recent study (Hutheyfa Abdulhussein Al-Salih and al.,2020332) . CPA can be associated with negative effects on endothelial cells (Toshio Ohtani and al.,2006333) and causes injury to the blood vessels (L. Zeng and al.,2010334) This could be explained by the interference of oxidative stress or the induction of pro-inflammatory molecules (Pragathi Duggina and al.,2015335) , This is what generates a hemorrhage . These effects " necrosis , cell lysis , hemorrhage " were observed in the tissue sections of CPA group . CPA is able to associated with the induction of hepatic metallothionein (Carmen Mufioz and al.,1990328) which leads to the sequestration of zinc (Nehal Abdelhamid and al.,2019329) . Which means that zinc deficiency can be the cause of these effects , Really , zinc had a positive effect on hepatic tissue . Where the histological

sections of Zinc S , ZnONPs V group showed a significant improvement . but , the histological sections of ZnONPs K group showed less improvement . Also , Zinc plays an important role in maintaining membrane structure and function and preventing metabolic physiologic derangements of the vascular endothelium (BERNHARD HENNIG and al.,1996336) . Which reduces the hemorrhage resulting from the CPA in histological sections of Zinc S , ZnONPs V group . AA is also associated with supporting and protecting endothelial cells by : stimulating endothelial proliferation, inhibiting apoptosis, scavenging radical species (James M. May and al.,2013338) . A very important study was conducted in 2016, in which it was shown that AA can modulate the levels of apoptosis " 74% " and necrosis " 32% " stimulated by CPA (Marcus Vinícius Oliveira Barros and al.,2016331) . We mentioned earlier that the cause of hepatic necrosis is acute cholestasis , intrahepatic cholestasis, for example, results from errors in the metabolism of bile acids (Halima Sultana and al.,2021339) Zinc may not have a direct effect on metabolism of bile acids .Zinc is a micronutrient that is essential to human health. Zinc plays a major role in regulating every phase of the wound healing process; ranging from membrane repair, oxidative stress, coagulation, inflammation and immune defence, tissue re-epithelialization, angiogenesis, to fibrosis/scar formation. With huge demands for improved wound care, we need a more thorough in-depth understanding of the molecular mechanisms in which zinc functions (Lin PH and al.,2017) .

4.2.Testicular tissue

The testicle is of great importance as a result of its competence in the spermatogenesis and production of male hormones , including the testosterone responsible for maintaining the process of spermatogenesis (Nabila I. El-Desouki and al.,2016) . spermatogenesis occurs inside the seminiferous tubes , where the diploid spermatogonia differentiate into primary spermatocytes, Then it gives two secondary spermatocytes. Each one produce haploid spermatids. Spermatid transforms into spermatozoa (Tung Nguyen Thanh and al.,2020) . We have previously shown that CPA induces oxidative stress in the testicles.

On the other hand, we mentioned that testicular cells are characterized by rapid division, such as cancer cells, which exposes them to the risk of CPA more , where phosphoramide mustard able to alkylate their nucleic acid , which causes its toxicity (Akram Hosseini and al.,2018) . CPA can also induce testicular toxicity by activating pro-inflammatory molecules like

; like NF-kB and MPO and inflammatory cytokines such as TNF-α and interleukins (Ashif Iqubal and al.,2020) .

As a possible result of the foregoing, it has been proven that the CPA is linked to spermatozoa abnormalities “head and tail” , which is reflected in its motility and fertility, and a severe decrease in its number (Maha Aly Fahmy and al.,2015) . Where CPA affects the motility of spermatozoa , for example through reduce Na-K-ATPase activity , which is highly sensitive to toxic stress (MA Rezvanfar and al.,2008) . And because ion balance in spermatozoa essential for sperm motility and fertility , there is a great need for activity Na,K-ATPase (Tamara Jimenez and al.,2011) . As for the number, CPA causes a decreased spermatozoa count by activating pro-apoptotic molecules “Bax and caspase-3” (Mustafa Cengiz and al.,2020) .

This was evident in the results of histological sections of the CPA group , where we noticed destroyed seminiferous tubes and low spermatozoa count . While the tissue sections of the zinc salt group showed a clear improvement , and there was a greater improvement in AA-ZnONPs group . And no improvement in the tissue sections of K-ZnONPs group .

The improvement in the tissue sections is due to the fact that zinc deficiency, for example, can cause testicular insufficiency and infertility (Laura M. Plum and al.,2010) . Zinc may prevent apoptosis by inhibiting the apoptotic protease, the caspase 3 or by promoting DNA synthesis and the anti-apoptotic proteins Bcl-2 activation (Amal Ibrahim El-Refaiy and al.,2012) . and more especially , ZnONPs might increase the function of mitochondria to decrease apoptosis inducing factor and cytochrome c which cause apoptosis , and then prevent cell apoptosis (Hoda H. Anan and al.,2018) . On the other hand , zinc served to ameliorate testicular toxicity by reducing inflammatory mediators “NO, iNOS, TNF- α” (Samir Abd El-Monem Bashandy and al.,2016354) . Zinc also able to reduce testicular oxidative stress (Ahmed M. Kabel ,2018) , as we have already explained, we can suggest this : Reducing oxidative stress reduces the pressure on Na,K-ATPase and restores it to its previous activity, being sensitive to oxidative stress .In a very interesting 2012 study, vitamin C was shown to reduce testicular toxicity by increasing zinc levels in the testicles (Oluseyi C Ayinde and al.,2012) .

What explains the great correlation between zinc and AA in reducing testicular toxicity. What can explain the increase in the percentage of improvement in ZnONPs V group . AA is also able to reduce the levels of pro-inflammatory molecules (Safaa Mohamed El Kotb and al.,2020) . zinc is a micronutrient that serves as a cofactor for more than 80 metalloenzymes involved in DNA transcription and protein synthesis.38 These above mentioned factors could change sperm quality and improve cryptorchidism outcome as was seen in our study. Many investigators showed significant decrease in diameter of seminiferous tubules and thickness of germinal epithelium in cryptorchid testis. The similar phenomenon was observed in our study, but administration of zinc could improve the height of germinal layer after 15 days.

Conclusion & future perspectives

Conclusion

Cyclophosphamide (CPA) is a great drug that is widely used in anti-cancer chemotherapy. However, the clinical use of Cyclophosphamide can also trigger dilated a toxicity in various organs including liver and testis. The biological synthesis of ZnNPs using ascorbic acid refers to their potential properties as natural stabilizers of nanoparticles, which characterized by different methods; UV-VIS and FTIR spectroscopies. Also we may be classify these compounds as one of the important biological and medical molecules that can used in pharmaceutical and medical fields In in-vitro study, results indicate the positive biological effect, especially anti-oxidants and anti-inflammatory activity proves that the zinc nanoparticles have high biological effects in reducing many diseases related to these mechanisms. In in-vivo study, the enhancements to the AA-ZnNPs through improvement in biochemical, hematological markers or oxidative stress confirm the effectiveness of these compounds in protecting rats from the toxic effects of CPA in liver and testis. Results indicate the ability of AA-ZnNPs treatment to rat's protection in the stability of cells against liver and testis injury caused by CPA. We shed the strong influence of ZnNPs in histological sections. This allows us to consider that these treatments have a potential effect on reducing Cyclophosphamide toxicity.

Future perspectives

Given the importance of these results, they open other perspectives, including:

- Evaluation of the protective effect of AA-ZnNPs against cancer disease.
- Carrying out other in-depth analyzes to know the effect of CPA on the DNA and the factors involved in it
- Evaluation of the effect of ZnONPs against reproductive and liver disease
- Explore and elaborate new drugs to reduce CPA induced liver and testis toxicity

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