Republic of Iraq

Ministry of Higher Education

and Scientific Research

Al-Mustansiriyah University

College of Pharmacy

Hormonal and Hematological Changes in Women in reproductive age with Toxoplasmosis Infection before and after Spiramycin Treatment

A thesis

Submitted to the Department of clinical laboratory sciences and the Committee of Graduate Studies of the College of Pharmacy/ Al-Mustansiriyah University in Partial Fulfillment of the Requirements for the degree of Master of Science in Pharmacy (clinical laboratory sciences)

By

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2015 A.D. **1437 A.H.**

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

وَقُلْ رَبِّ زِدْنِي عِلْمًا

صدق الله العظيم

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Dedication

To ...

My parents

For their endless love,
support and encouragement

My beloved Husband

&

My all dear Family

Raghda

Acknowledgments

Praise is to almighty ***Allah*** *gracious for enabling me to finish what I started and for helping me to present this work.*

I would like to express my profound indebted and appreciation to my supervisor ***Assist. Prof. Dr. Jaber Hameed Hussien*** *for his valuable scientific guidance, discussion and suggestion throughout my work.*

I would like to express my heartfelt gratitude and appreciation to my supervisor ***Assist. Prof. Dr. Mohammed A. Al-Aaraji*** *for his valuable advices throughout the course of my work.*

My deepest thanks with respect to ***Dr. Monther Faisal Mehdi & Dr. Inaam S. Arif,*** *for her valuable supports and encouragement.*

This work would never have been completed without the encouragement, valuable supports and scientific advices from ***My Mother and My Teacher Dr. Ilham Albasri,*** *my special thanks to her.*

My deepest thanks to ***Mr.Taha Hussien*** *for his scientific advices during my work in ALKUT Maternity hospital.*

I am honestly grateful to ***Dr. Haitham Qasim & Dr. Huda Jaber*** *for their help throughout my work.*

My deep gratitude to ***Directorate of health Wasit*** *&* ***AlKUT maternity hospital,*** *for offering the opportunity to continue my postgraduate study.*

Iam honestly grateful to my colleagues ***Noor Moneer, Hadeel Fadhil*** *for their friendship, valuable support, and constant help through the course of this work.*

I would like to express my deep gratitude for ***my husband Dr.Sarmed Al-Qutby*** *for his patience and huge support during my work.*

Finally, I am really grateful to all people who helped me even by their encouragement and prayers.

Raghda

List of Contents

Chapter Two: Materials & Methods

List of Tables

List of Figures

List of Abbreviations

Abs	*Antibodies*
AF	*Amniotic fluid*
Ag	*Antigens*
AIDS	*Acquired immune deficiency syndrome*
CLIA	*Chemiluminescent immunoassay*
CMI	*Cell mediated immunity*
CNS	*Central nervous system*
CO2	*Carbon dioxide*
CSF	*Cerebral spinal fluid*
dL	*Deciliter*
E2	*Estradiol*
ECL	*Electrochemilumenisce*
ECLIA	*Electrochemiluminescent immunoassay*
EDTA tube	*Ethylenediaminetetraacetic acid tube*
EIA	*Enzyme immune assay*
ELFA	*Enzyme linked fluorescent assay*
ELISA	*Enzyme linked immunosorbant assay*
FSH	*Follicle stimulating hormone*
G	*Gram*
GnRH	*Gonadotropin realsing hormones*
Hb	*Hemoglobin*
hCG	*Human chorionic gondotropin*
HPA	*Hypothalamus-pitutary-adrenal*
HPG	*Hypothalamus-pitutary-gonadal*
IgG	*Immune globulin G*
IgM	*Immunoglobulin M*

IMI	*Immature myeloid information*
L	*Litre*
LH	*Luteinizing hormone*
LSD	*Least significant difference*
Mg	*Microgram*
mIu	*Micro international unit*
ml	*Millilitre*
NBT	*Nitroblue tetrazolium stain*
Ng	*Nano gram*
NRBC	*Nucleated red blood cells*
PCR	*Polymerase chain reaction*
PCV	*Packed cell volume*
Pg	*Picogram*
PMNs	*Polymorph neaucler neutrophils*
PRL	*Prolactin*
PSA	*Pyrimethamine, sulfadiazine and folic acid*
RBC	*Red blood cell*
Rpm	*Round per minute*
SAS	*Statistical Analysis System*
TSA	*Tachyzoite soluble antigen*
VIDAS	*Viteck immune diagnostic assay*
WBCs	*White Blood Cells*
µL	*Micro litre*

ABSTRACT

Toxoplasma gondii is the causal agent of Toxoplasmosis which infects a large proportion of the world's population, especially pregnant women; it may cause abortion and severe damage to the fetal central nervous system. This study was conducted to detect the influence of acute *T.gondii* infection on some hormonal levels (estrogen, progesterone, FSH, LH and prolactin). As well as, the effect on some hematological parameters WBC, Hb, PCV , Platelets, and the phagocytic activity of neutrophils by using (NBT) stain in pregnant (first trimester) and non pregnant women infected with Toxoplasmosis. This study includes 64 infected and 24 healthy women, attended Al-kut Maternity Hospital/Wasit province from September 2014 to May 2015 and giving the infected women spiramycin treatment for 2 months. The results showed that infected women with acute toxoplasmosis always associated with high concentration of both estrogen and FSH (518.67 ± 34.42 pg/ml) (0.735 ± 0.24 mIu/ml) respectively for pregnant, and (132.37 ± 9.94 pg/ml) (9.08 ± 3.19 mIu/ml) for non pregnant compared to healthy controls. LH hormone associated with high concentration only in non pregnant infected women (11.06 ± 3.95 mIu/ml), whereas no significant changes were recorded for Progesterone and prolactin.

The NBT test revealed a significant increase in infected women compared to post-treated and control group in pregnant and non-pregnant groups. Significant decrease in Hb, PCV and platelets count were detected in pregnant infected women compared to their control. While in non pregnant infected women showed significant increased in WBC and platelets count only. While there is significant decrease in PCV. We conclude that acute *T. gondii* infection is associated with variations in levels of serum hormones and these variations may influence the immune system and thus increase the susceptibility to Toxoplasmosis infection.

Chapter One

Introduction

1.1 Toxoplasmosis infection:

Toxoplasmosis is a parasitic disease caused by *Toxoplasma gondii*, which described and named by Nicolle and Manceaux for the first time in 1908. They found this parasite in a tissues of hamster called (the gundi)[1]. At the same time; *Toxoplasma gondii* was identified by Alfonso Splendore in a rabbit in Brazil [2] .The organism was named *Toxoplasma gondii*, due to its shape. The 'Toxoplasma' comes from the Greek word 'toxon' which mean bow, that describe crescentric shape of tachyzoite, and the species name 'gondii' from the rodent from which it was first isolated [3]. The first researcher who discovered the *Toxoplasma gondii* in human was janku in 1923. He observed tissue cysts on retina of a child suffering from hydrocephalus [4, 5].

In 1927 Torres reported the presence of *T.gondii* in many tissues in human as well as the nervous system [6]. In 1939; Wolf et al. successfully isolated the parasite from tissue from a neonate with encephalitis, by animal inoculation [4]. This considered the first example of an organism causing disease in utero. In 1948 Albert Sabin and Harry Feldman developed the Dye test in which estimated the prevalence of chronically infected individual [7]. In 1952 Wilder had the ability to demonstrate *T. gondii* in adult eye tissues (Toxoplasmic retinochoroiditis) [8]. After about two years; in 1954 Jacobs et al. isolated the parasite from the eye. Whereas in 1956 Frenkel described the reactivation of a chronic infection in man [9]. Many years later, the full life cycle of parasite was described in 1970, by the research that discovered a sexual stage in the cat's small intestine [10]. Machattie was the first who recognized the parasite in Iraq in 1938, in two street dogs smear from their spleen and lungs in Baghdad [11].

1.2 Life cycle:

Toxoplasma gondii is an obligate intracellular parasite that has a complex life cycle that includes sexual and asexual replication [12].These two-stage life cycle, consisting of a sexual phase in the (cats) which consider the only definitive host, and an asexual phase occurring in the intermediate host, intermediate hosts are probably all worm-blooded animals , including humans, cats, dogs and birds [10].

Human acquired *T. gondii* infection by ingesting of the tissue cyst stage that found in inadequately cooked meat of infected animals, or by ingesting of food that contain oocyst stage that passed from infected cat feces [13] .

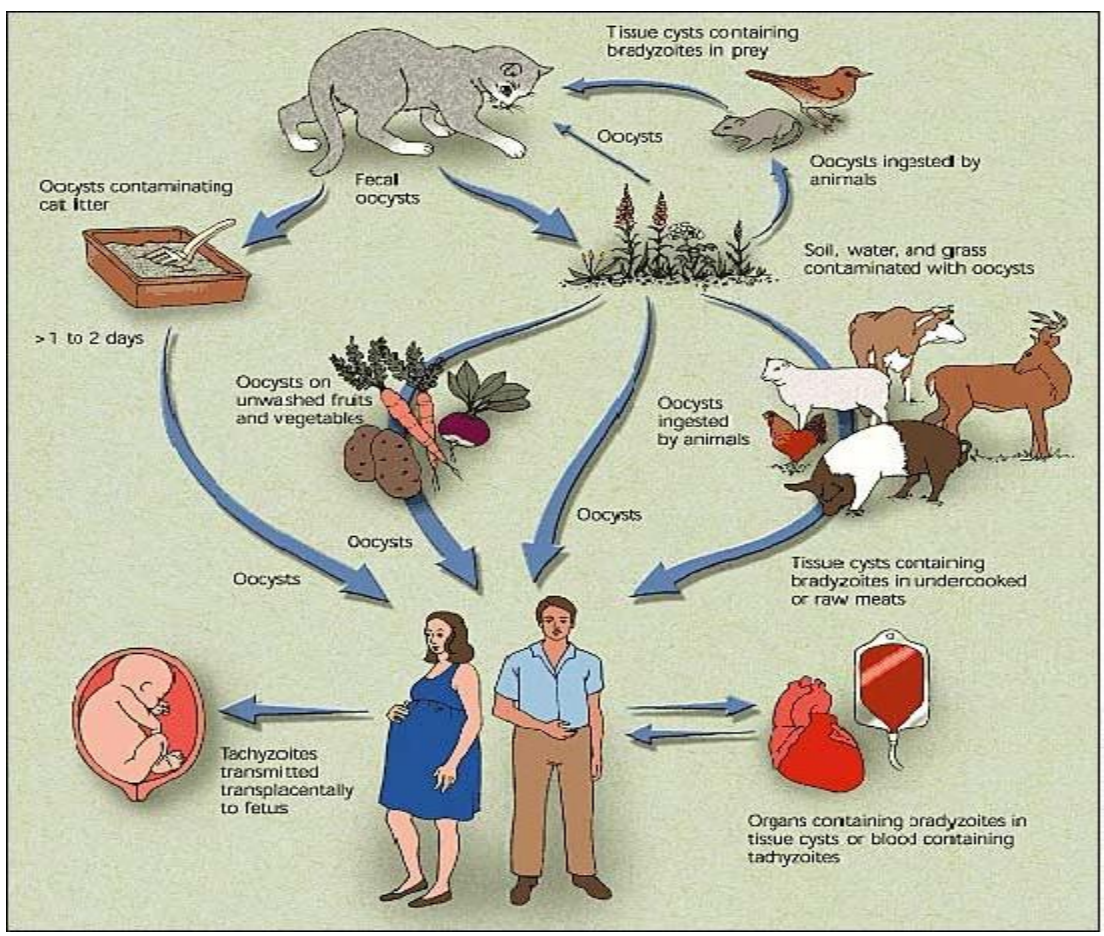

Figure (1-1) [14]: Pathways for *Toxoplasma gondii* infection.

All cat family members considered the main reservoirs of infection because they are the only known definitive hosts for the sexual stages of *T. gondii.* They become infected by consuming an infected tissue cyst. The *T.gondii* pass successfully through the stomach of the cat, subsequently reach the small intestine and infect the epithelial cells, these epithelial cell provide good environment for sexual development of the parasite; results in production of zygote containing cysts in a large number which is called (oocyst) [(14)]. The infected epithelial cells will rupture and spread the oocyst in intestinal lumen then passed into cat's feces to the environment result in contamination of water, food and soil, all these events known as the sexual cycle [(15)].

After shedding of oocyst, takes about 5 days to spouraltion and become infective [(13)]. It takes about 1-2 weeks, during this period a huge number of oocysts may be shed. Oocysts have the ability to live for several months in the environment and resistance to freezing, drying and disinfectant. Also Oocysts are known to survive on fruits and vegetables for long periods [(15)]. Cats become immune after the primary infection and if the cats become reinfected again they do not shed oocysts and the immunity may persist for about 6 years. The shedding of oocyst may be re-established in some cases if reinfection occurs again, but less than the primary infection [(16)]. Human acquired infection by several ways, either by ingestion of raw infected meat, ingestion of inadequately cooked food which contains some oocyst , or some times from the mother to fetus through the placenta [(17)].

The asexual life cycle starts by the ingestion of meat containing tissue cyst by human and bird or any warm-blooded animal. The oocyst degraded by the gastric juice, and the bradyzoites are released in the intestine. Then bradyzoites converted to tachyzoites in the small intestine

and spread to the circulation within 15 hours. Tachyzoite rapidly multiplied in any cell of the intermediate host while in definitive host multiplies in non intestinal epithelial cells [(18)]. Tachyzoites multiply asexually within the host cell by repeated endodyogeny and two progeny form within the parent parasite [(19)]. Replication eventually results in fresh tachyzoites and infection of neighboring cells. As the immune response increase, lead to conversion of the tachyzoite into the bradyzoite stage [(2)]. The tachyzoite stage is found during the acute phase of toxoplasmosis [(20)]. Differentiations into bradyzoites lead to form tissue cysts that first appear 7 to 10 days post infection. Bradyzoites also called (cystozoites) undergo slowly multiplication in the tissue cyst. When the bradyzoites divided by endodyogeny the tissue cysts grow and remain intracellular [(18)].

These cysts can be found mainly in the eye, CNS, skeletal, heart muscles, and placenta where they may remain in host for a life time. When tissue cyst begins proliferation all over the body this mean that represent the asexual cycle (chronic phase).Eating tissue of infected animal containing cysts result in cysts rapture when passed through the digestive tract, lead to release of the bradyzoite, which will infect the epithelium of the intestinal wall, then this will be change rapidly to tachyzoite stage, this tachyzoite will spread throughout the body, at this point the asexual cycle is completed [(20)]. Tachyzoites are more susceptible to destruction by proteolytic enzymes than bradyzoites. The bradyzoites remain orally infective because have resistant to gastric digestion, but tachyzoites can be destroyed by gastric juice [(18)]. If the host immune response is compromised, such as in AIDS patients the bradyzoites can reactivate and convert to tachyzoites [(21)].

Toxoplasma gondii is the only known apicomplexan that can transmit directly orally among intermediate hosts and cause infection

without cycling through its definitive feline host and this provides it with the potential for clonal spread among wide range of intermediate hosts [22].

1.3 Strains:

Toxoplasma gondii has three types of strains which differ in virulence and epidemiology[23]. Strains of *T.gondii* exhibit virulence differences. A previous studies conducted on mice have reported that infections with the different clonal lineages of this parasite result in very different outcomes. Type I strains are highly virulent; whereas type II and type III are relatively non virulent[24]. Type I mostly caused severe congenital and ocular disease in humans, and considered more pathogenic than other strain[25]. Type II and III strain parasites cause non lethal infection and lead to a chronic latent infection of brain and muscular tissue [21]. While Type III has been shown to be more common in animals. However, *T. gondii* was considered to have little genetic variability. Several studies in Brazil on isolation of *T. gondii* show that the strain of parasite in USA and Europe from strain of Brazil genetically and biologically [23].

1.4 Epidemiology:

Toxoplasma gondii has a worldwide distribution, but its prevalence varies greatly, and also varies within the same countries .Toxoplasmosis is widespread infection affecting large number of population around the world, with infection rate 12% - 90%. This percentage may increase with age, sanitary habits, low education, crowding, consumption of inadequately cooked meat and contamination with animals [26].

The climatic conditions play important role in spread of infection, since surviving of oocyst occurred in warm and moist soil rather than hot

and dry soil [27]. Higher prevalence is found in areas with hot and moist climate, i.e. tropical countries while those with lower prevalence occurred mostly in country with colder climate. However there is others factors affects on seroprevalences variations in human, such as, dietary habits, social factors, sanitation and health education[28]. Also high seroprevalence is found more in those with older age group, but this varies in different country and in different social level. So in children whom lived in bad sanitary conditions like water contamination; the seroprevalences may reach maximum levels [29, 30].

Many countries have high seroprevelnce rate approximately reach 75% such as France and Germany, as well as the rate of seroprevelance in women of childbearing age may reach greater than 50% in Africa, America and western Europe[25]. During pregnancy the rate of maternal *T. gondii* infection ranges from about 1-310 cases per 10,000 pregnancies in Europe, Australia, Asia and Americas. While prenatal *T.gondii* infection in these countries 1-120 cases per 10,000 births [31].

Recent seroprevalences of *T.gondii* is 0.79-12.9% in Korea recorded by Shin [32], and 36% In Turkey [33]. While the seroprevalence rate of toxoplasmosis among the general population in Iran was 39.3% [34]. Another study in India suggests a prevalence rate 20–40% in India [35], And around 40 % in Egypt [36]. While in Jordan, the rate of toxoplasmosis was 31, 6% among pregnant women reported by Jumaian [37].

1.4.1 Toxoplasmosis in Iraq:

A study in Kurdistan area in 1992 showed that the prevalence in aborted women was 15.62 %[38]. Another study in 1998 recorded that, the prevalence of infection was 9.4% in age 16-20 years [39]. Whereas a study carried out in Basrah (2006) showed that the prevalence of toxoplasmosis was 41.1-52.1% [40]. Karem in (2007) revealed that the infection rate was 32.1% in aborted women in Baghdad province by using ELISA [41]. Al-Rawi recorded that the *Toxoplasma* infection rate in 2009 was 4.16% and 15.83% in aborted women in Baghdad by using both of ELISA and PCR [42]. Then in 2010, Al–Shikhly found that females with age ranging from (15-19) and (20-24) years showed 30.63% prevalence rate of infection with *Toxoplasma* by ELISA IgG in Baghdad[43]. Afterward in 2011 Al-Mayahi found that the rate of Toxoplasma infection In Kut province was 66.35 % IgM , 44.71% IgG in women using ELISA technique[44]. Al-Saadii in (2013) found that the ratio of infection in males ageing between (18-57) years was 30.25% IgG by using ELISA technique [6].

1.5 Pathogenesis of Toxoplasmosis:

Human acquire infection mainly by consumption of infected meat containing cysts or contaminated food containing oocysts from cat feces. After entrance of contaminated food with sporoziote or oocyst into the intestine, Bradyzoites or the tissue cysts which come from sporoziote or oocyst will enter into the intestinal epithelial cells and proliferate there [45]. Then the cysts reach the mesenteric lymph nodes then to different organs of the body through lymphatic and blood vessel [46].

As a result, necrosis may occur in intestine and lymph nodes and then spread to the other organs of the body and the symptoms depend on degree of necrosis especially when injury occurred in important organ

such as heart, eye and adrenals. Tissue death may occur due to destruction of host cell when tachyzoite exit from these cells, and not as a result of a *Toxoplasma* toxin. Clinical manifestation of toxoplasmosis are asymptomatic, only 10-30% of infections are symptomatic, the symptoms are lymphadenitis and lymphadenopathy. These clinical symptoms occurred mostly in immmuno-compramised patients and in congenital disease [(47)].

In pregnant infected women with toxoplasmosis, the tachyzoite will spread through the placenta to the fetus either during pregnancy or at birth[(48)]. *T.gondii* has the ability to infect all kinds of cells by active process. Then it starts to form a parasito- phorous vacule that does not blend with intracellular organelles [(45)]. Approximately after three week of infection, the tachyzoites begin to localize in muscular and neural tissues (as tissue cysts) and disappear from visceral tissues; tachyzoites may remain in the nervous system (brain and spinal cord) for a longer time [(49)].

1.6 Symptoms of Toxoplasmosis infection :

The symptom of *T.gondii* infection depends on the age and immune status of the patient.

1.6.1 Primary infection :

Primary infection of toxoplasmosis is often asymptomatic in healthy adults. But some, may experience mild flu-like symptoms: including myalgia or enlargement of lymph node, that typically resolve over several weeks.

Young children and immunosuppressant people, such as those taking certain types of chemotherapy, or those with HIV/AIDS, may have severe toxoplasmosis symptoms, result in brain or eye damage. The organism is transmitted transplacentally to fetus (Congenital infections)

may result in miscarriage or born with sign of toxoplasmosis infection (e.g., jaundice, microcephaly, macrocephaly, seizures). However, infected infants are often asymptomatic at birth [50].

1.6.2 Latent *Toxoplasma* infection:

Latent toxoplasmosis is usually asymptomatic, and life-long infection, in which host become infected without suffering any symptoms. It is characterized by the presence of bradyzoite cysts in the muscular or nervous tissues, and this will cause lifelong protection and immunity to reinfection, represented by the presence of low levels of IgG in the serum of infected host [51, 52].

1.7 Laboratory diagnosis:

Various laboratory techniques are performed in diagnosis of toxoplasmosis that is either indirectly (antibodied detection) or directly, (parasite detection or its DNA) [54].

1.7.1 Direct methods:

1.7.1.1 Histological diagnosis:

Diagnosis by this method performed by finding parasite in host tissue through using biopsy to remove the *T.gondii* from tissue. This approach is mainly helpful in immuno- suppressed patients with AIDS, because of having low antibodies (Abs) and delay in the synthesis of the Abs. Toxoplasmosis is a common cause of lymphadenopathy but tachyzoites are not usually found in biopsy specimens from lymph nodes although characteristic histopathologic features can support the diagnosis[53]. Also *T. gondii* can be detected through aspiration from the brain or in tissue biopsy or from cerebrospinal fluid (CSF) [54].

1.7.1.2 Isolation of *T.gondii* :

Inoculation of blood or body fluids such as CSF, amniotic fluid (AF) and brain aspirate into tissue culture or intra peritoneally or intra cerebrally inoculation into mice, then, the exudates examined for tachyzoites after (8-10) days [45].

Isolation of the organism from the placenta in neonate is a diagnostic tool for congenital Toxoplasmosis [55].

1.7.2 Indirect Methods :

1.7.2.1 Skin test (delayed hypersensitivity test):

This test is used to measure the cell mediated immunity (CMI). It is one of the useful population surveys in epidemiological studies as a screening test [53].

In 1948, Frenkel employed it for the first time, which includes injection intradermally of soluble antigen (toxoplasmin) from a pathogen. The positive test demonstrates past infection with that pathogen, but the negative result does not always indicate the absence of antibodies or infection [55].

1.7.2.2 Serological tests :

The initial and primary method of diagnosis of *T. gondii* is the serologic tests which is use for demonstration of specific antibody. There are many serologic tests used to measure different antibodies that possess unique patterns of rise and fall with time after infection [58].

1.7.2.2.1 Sabin-Feldman Dye test (DT):

This test was described by Sabin and Feldman in 1948, it is the "gold standard" test, which is sensitive and a highly specific test. This test has disadvantages because of its high cost and the risk of infection because they use a live and virulent *T.gondii*. This test involves incubation of tachyzoites and a complement-like accessory factor and the host serum, and then methylene blue dye was added to the mixture [(56)]. The test is based on the presence of certain antibodies that avoid entering of methylene blue dye to the cytoplasm of toxoplasma organisms.

If antibodies are present in the serum, the tachyzoite will not stained by the dye so appears colorless (indicate positive result) if there is no antibody the tachyzoite stained and appear blue (that is, a negative serum sample) [(56)].

1.7.2.2.2 Indirect Fluorescent antibodies test (IFAT):

This test first described in 1957 by Goldman for detection of *T.gondii* . The test involves using of antiglobulins labeled with fluorescent dyes, after excitation by ultra violate light the fluorochrome emit visible light. Detection of antibodies via this method act mainly against the cell wall of the parasite like to antibodies in Sabin-Feldman dye test but without involve live parasites [(57)].

Both IgM and IgG can be detected in this method, when monoclonal Abs is used. In comparison with the dye test this test considered simple and easy to use [(58)].

1.7.2.2.3 Complement fixation test:

This test is used for determination IgG and IgM. Its can detect antibodies from beginning of infection till 2 years. Although it is a basic method in diagnosis but It is not often used in routine work where the detection of IgG by means of Enzyme immunoassay (EIA) tests is widely used[59].

1.7.2.2.4 Latex agglutination test (LAT) :

This test performed easily, inexpensive and sensitive to be used as a screening test and allows visual observation of the antigen-antibody complex [60]. U shaped well microtiter plates are used, then adding formol-fixed *Toxoplasma* tachyzoites after dilutions of sera. Positive samples (make agglutination), whereas negative samples (make precipitation). The test is simple and easy to do although required large amounts of antigen [56].

1.7.2.2.5 Dipstick Dye immunoassay (DDIA):

This test can detect IgM and IgG antibodies against toxoplasmosis in human sera. This assay involve conjugation of blue dye particles with sheep anti-human IgG and rabbit anti-human IgM and soluble antigen of tachyzoites (TSA) of *T. gondii* RH strain as the detective antigens. This test need only 15minutes and it agrees with ELISA test and are especially suitable for field application [61].

1.7.2.2.6 Enzyme Linked immunosorbent assay (ELISA):

This test is simple, fast and sensitive for quantitative determination of unknown antigen and antibodies, based on the uses an enzyme

conjugated with antibody or antigen as a marker for the detection of an antibody or antigen [6]. There are two method can be used either detected the presence of antigen or detect the antibody. This test depends on color changes of the end product of reaction catalyzed by the enzyme to identify a substance [62]. ELISA test considered the simplest technique used for detection of *Toxoplasma*-IgM antibodies in infected patients [63].

1.7.2.2.7 Enzyme linked fluorescent assay (ELFA):

This assay considered simple, quick and accurate method used to measure IgG and IgM antibodies to *T.gondii*. All the steps of this method can be performs by the VIDAS machine automatically [6].

The principle of this technique includes a two-step, enzyme immunoassay sandwich method combined with fluorescent detection in the end of the reaction. This method has a high specificity for anti-*Toxoplasma* IgM and IgG detection [64].

1.7.2.2.8 Automated testing methods :

Involve many methods such as, chemiluminescent immunoassay (CLIA), and electrochemiluminescent immunoassay (ECLIA). Because of its high sensitivity and specificity, it's used for routine screening work. These methods recognize *toxoplasma* infection either by detection of IgG antibodies specific for the parasite, or detection of IgM antibodies which is helpful to differentiate between past and recent infection, use of IgG avidity test in case that IgM remain for several months for determination the time of infection [65].

Cobas e 411 machine is based on Electrochemiluminescence technique have the ability to detect anti-*Toxoplasma* IgM antibodies

during the early stage of acute infection better than ELISA and ELFA technique, and confirmed the usefulness to diagnose acute Toxoplasmosis during the first trimester of pregnancy[165].

1.7.3 Molecular Diagnosis:-

Polymerase chain reaction (PCR) method is used for detection of *T. gondii* DNA in body fluids (urine, cerebral spinal fluid, amniotic fluid, blood, ocular fluid) and tissues samples (brain tissues and bronchiolar lavage fluid)[66].

Polymerase chain reaction performed on amniotic fluid is helpful for early diagnosis of fetal *T.gondii* infection. This way the fetus will not be exposed to risks as a result of complex tests that may hurt the fetus. The test could be performed after (18) weeks of gestation. It has specificity of (100%) [67]. So PCR represent the gold standard examination for diagnosis of fetal Toxplasma infection [68].

1.7.4 Avidity test:

There are many tests for measuring the avidity of toxoplasma IgG Abs to discriminate between latent infection and acute infection by measuring the affinity (avidity) of IgG that bind to *T.gondii* antigens. So in acute infection IgG bind to antigens of *T.gondii* weakly its considered (low avidity) and in chronic infection the IgG binding strongly to the antigens (high avidity) [6]. The IgG avidity test used to detect the approximate time once the initial infection occurred; which is useful to know the phase of infection and so for good management during pregnancy [69].

Avidity test is helpful in screening pregnant women and follow-up with reduction the screening costs by avoid subsequent costly investigation for both mother and fetus also unnecessary treatment [70].

1.8 Treatment :

The treatment should be started when the presence of the parasite is confirmed to avoid the transmission of the parasite from mother to the fetus [67]. Drugs used for treatment based on time of diagnosis which includes:

1.8.1 Pyrimethamine:

Widely used for therapy of toxoplasma infection, it is a folic acid antagonist, may block absorption of the vitamin B9 (folic acid), especially when taking high doses over a long period. So usually given in combination with folic acid[28]. Sometimes thrombocytopenia or leucopenia may occur as side effects. It has an effective action in the acute stage but its disadvantage; it will not eradicate the infection [28].

pyrimethamine have other side effects which is bone marrow suppression and liver toxicity. This side effect is risky for immunocompromised patients and because of teratogenic effect, it is not recommended during pregnancy especially in the first trimester of gestation. Pyrimethamine usually given in synergistic combination with sulfadiazine for *Toxoplasma* infection treatment [71].

1.8.2 Spiramycin :

Spiramycin is a macrolide antimicrobial agent often used for toxoplasmosis treatment during pregnancy and congenital toxoplasmosis. Because spiramycin has been not found teratogenic and safe for use in the

pregnant women, fetus, and newborn. Spiramycin acts by reducing the transmission of the parasite from the mother to the fetus[72]. In case of establishment of fetal infection, treatment with spiramycin should be replaced by PSA (pyrimethamine, sulfadiazine and folic acid) after the 18th week of gestation [73]. Since this macrolide does not cross placenta, it is not suitable for treatment of fetal infections [74].

1.8.3 Clindamycin :

This drug is quickly absorbed and diffuses well in the CNS therefore; it was used as an alternative to sulfadiazine If patient has a allergy reaction to sulfa drugs, so pyrimethamine plus clindamycin can be used instead[75].

It is rarely used for treatment of congenital infection or primary maternal infection during pregnancy due to the ability to penetrate fetal blood when given to pregnant women. Clindamycin may cause ulcerative colitis which considered the main side effects of this drug [76].

1.8.4 Azithromycin:

Azithromycin is a semi-synthetic macrolide antibiotic which is useful in early infection of *Toxoplasma gondii* and also given as prophylaxis and used as an alternative drug for congenital toxoplasmosis cases because of its action in reducing the rate of proliferation rate of *T. gondii* [77].

This drug is effective against tachyzoite and cyst forms of the *T.gondii* [77]. Also it can be used safely during all trimesters of gestation and in immunocompromised patients [78].

1.9 Influence of hormones on *T.gondii* infection:

There is significant evidence that the course of *Toxoplasma* infection in both humans and animals is affected by the steroid hormones levels. Several studies demonstrated the effect of sex hormones on the course of *T.gondii* infection because of the capability of the parasite to cause congenital disease in the fetus if the infection acquired during pregnancy. The first study was done on mice by Henry and Beverley in 1976, which demonstrated the differences in inflammatory and immunity response between females and males mice after *Toxoplasma* infection. These studies demonstrated that the male mice will have a mild inflammation of the brain in comparison to female mice which develop severe symptoms[(79)].

Additionally, other experiments on mice demonstrated the role of sex hormones and found that administration of estrogen had exacerbated disease in mice while gonadectomy increased resistance against the disease. Similar finding in guinea pigs, when infected with *Toxoplasma gondii* followed by estrogen administration, lead to increased mortality and exacerbate infection, due to a severe depression of cell-mediated immunity caused by the effect of high doses of estrogen comparing with non treated control animals [(80)]. lymphadenopathy considered as possible clinical symptom although many cases of Toxoplasmosis infection are asymptomatic in healthy humans. A previous study, found that signs and symptoms of the disease varied according to age and gender in spite of infection incidence was similar in both males and females. In mature adult, lymphadenopathy was more frequently appeared in females whom over 25 years, while those below 15 years of age males develop lymphadenopathy more frequently than females in the same age group[(81)].

All these studies support that there is a direct role in female sex hormone and the severity of *T.gondii* infection by affecting the immune system [(81)].

The common hypothesis for immunological differences between male and female is the influence of sex hormones on the immune system, especially estrogen, progesterone and testosterone. A previous study by Klein SL [(82)] shows that there is a significant association of immune system and endocrine systems and these in turn affect the target genes in immune cells due to the presence of sex hormone receptors inside immune cells including: lymphocytes, macrophages, granulocytes, and mast cells.

1.10 Hormonal changes during pregnancy:

Pregnant women develop changes in levels of some hormones including estrogen which is produce mainly by placenta, progesterone which is produce mainly from corpus luteum and placenta, human chorionic gonadotropin (hCG) also produced from the placenta and prolactin produce from pituitary gland .

Suppression of menstrual cycle occurred in pregnancy is due to increase of progesterone and estrogens hormone which will cause suppression of the hypothalamic axis. Progesterone causes relaxation of uterine smooth muscle so prevent the rejection of the fetus[(83)]. Although these changes of hormonal and immunological nature are necessary to support a healthy pregnancy and prevent rejection of fetus, but it also has dramatically affect for the susceptibility to parasitic infection [(84)]. Maternal hormones during pregnancy cause alteration in the immunity of the mother because of fetal antigens. So reduction of pro-inflammatory response lead to alteration and susceptibility to infection will predispose

to Toxoplasmosis infection [84]. The prevalence of *Toxoplasma* infection increases after the third month of pregnancy due to elevation of progesterone and estrogen levels [85].

1.10.1 Estradiol (E2):

Estradiol or 17β-estradiol (E2), considered the primary female sex hormone . The name came from the estrous cycle due to its significant role in the regulation of female menstrual cycles, (E2) synthesized in breast, ovary, endometrial tissue, and brain. It is essential for the growth and differentiation of the sexual organs and development of breast tissue [86].

Estradiol in female serves as a growth hormone for the reproductive organs tissues, which support the endometrial gland with cervical glands, lining of the vagina and fallopian tubes. It's considered essential to maintain cyst in the ovary. During the menstrual cycle, estrogen is produced by the growing follicle and this is controlled by positive feedback mechanism through the hypothalamic pituitary axis which will cause increase in luteinizing hormone. This is called luteinizing hormone surge, and this will stimulate ovulation [87]. In the second half of the cycle (luteal phase), both hormones (estrogen and progesterone) changes the endometrium which will make it more appropriate for implantation. If pregnancy occurred there will be increase of estrogen that is produced from the placenta. A previous study showed that when administered estrogen will cause increase the possibility of acquiring the *Toxoplasma* infection [88].

1.10.2 Progesterone:

Progesterone is a female steroid hormone involved in menstrual cycle, pregnancy and embryogenesis of humans. Progesterone is one of the (progestogens) which is group of steroid hormones [89]. It represents the main progestogen in the body. It is also called the hormone of pregnancy, because of the critical role in supporting the endometrium and hence on survival of the conception, it has a vital role as a neurosteroid in brain function. Progesterone and other progestins also potentially inhibit secretion of the pituitary gonadotropins lutenizing hormone (LH) and follicle stimulating hormone (FSH), which lead to prevents ovulation from occurring during pregnancy [90].

Importance of progesterone to conception:

- Progesterone prepares the uterus for implantation by changing the endometrial glands to secretory stage. Also progesterone making vaginal epithelium thick to prevent penetration of the sperms. Progesterone diminishes the tropic effects of estrogen [91]. Progesterone levels will decrease result in menstruation in case pregnancy did not happen, normal menstrual bleeding is progesterone-withdrawal bleeding.
- Progesterone may decrease the maternal immune response during implantation and gestation to prepare the body to accept pregnancy.
- The effects of progesterone on uterine smooth muscle by inhibiting contraction [92].
- Progesterone inhibits lactation during pregnancy. But after delivery when progesterone levels falls, it triggers milk production.

- A fall in progesterone levels is possibly one step that facilitates the onset of labor.

In a previous study reported by Gay-Andrieu F *et al*, who study the infected macrophage by *T.gondii*, found that there is no obvious effect of progesterone on replication of this parasite[93]. Another study by Aiumlamai S *et al.* done on sheep during pregnancy, found that after infection of sheep by *T.gondii*, the level of Progesterone are reduced [94].

1.10.3 Luteinizing hormone & Follicle stimulating hormone (LH, FSH):

Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are large glycoproteins called (gonadotropins) because these hormones stimulate the gonads (the testes) in male and (the ovaries) in female. The LH & FSH are essential for reproduction; they are synthesized and secreted from (gonadotrophs) which is cells that is found in the anterior pituitary [95].

Luteinizing hormone stimulates sex steroids secretion from the gonads in both male and female. In females, LH induces ovulation of mature follicles on the ovary by secretion large burst of LH known as (the pre ovulatory LH surge).Within ovulation, the residual cells proliferate to form corpus luteum that produces progesterone for preparation of endometrium to a possible implantation . Thus LH is necessary to keep luteal function in the first two weeks of the menstrual cycle, in case of happening of pregnancy . The levels of LH will decline, and the human chorionic gonadotropin (HCG) hormone will function instead the luteal function[95]. When the follicle matured, the progesterone

level will increase lead to inhibition of estrogen production, result in decrease estrogen-negative feedback of gonadotropin realsing hormones (GnRH) in the hypothalamus which in turn lead to anterior pituitary stimulation and LH release [(96)]. Follicle stimulating hormone (FSH) and (LH) are responsible of regulate reproduction of the human body [(87)]. The FSH hormone essential for development of immature follicles in the ovary, and has important role in saving the small follicles from apoptosis. During the luteal-follicle phase FSH level will increase and reach peaks at about day three of menstruation due decline in the serum levels of progesterone and estrogen ,so no longer inhibit the release of FSH[(95)].

A study by Oktenli *et al*. illustrate that "acute *Toxoplasma* infection may cause temporary hypogonadotrophic gonadal insufficiency regardless of the course of the disease" [(97)].

1.10.4 Prolactin (PRL):

Prolactin (PRL) is a polypeptide hormone play important role in milk production in female mammals, synthesized and secreted mainly by anterior pituitary gland, in response to estrogen therapy, mating, food consumption, nursing and ovulation [(98)]. Prolactin has also a vital role in immune system functions, development of pancreas and in stimulation of the mammary glands to produce milk (lactation) which is normally starts at the end of pregnancy when the levels of progesterone fall and a presence of suckling stimulus. Prolactin also has been found to play an important role in maternal behavior [(99)]. During pregnancy, PRL involved in immune tolerance of the fetus by the mother. At the end of the pregnancy, PRL also contributing to pulmonary surfactant synthesis of the fetal lungs, moreover it promotes neurogenesis in maternal and fetal brains [(100)].

A previous study by Benedetto N *et al.* state that prolactin may limit the growth of *T. gondii* in murine microglial cell *in vitro* [101]. Another study by Dzitko *et al.* in 2008 state a high level of prolactin may be one of the important factors that preventing Toxoplasmosis infection in women[102]. While in 2010, 2012 the same author conclude that prolactin restricted the growth of *Toxoplasma* in the cells of mice and also human cell [103, 104].

1.11 Phagocytic activity of Neutrophils by using Nitroblue tetrazolium stain (NBT):

Neutrophils are the most common types of leukocyte that play a key part of the innate immune system. They are circulating phagocytes. The bone marrow responsible of production of neutrophils and they rapidly accumulate at the sites of microbial pathogens invasion, in *Toxoplasma gondii* infection , with half life about 6 to 9 hours. Neutrophil represent about 85% of circulating phagocytes and more than 50% of the total circulating white blood cells. Large numbers of neutrophils originate in the tissue this will enable them to mobilize rapidly in response to onset of inflammation or infection where they phagocytes invading bacteria [105].

The immune response to *Toxoplasma gondii* infection is complex which involves both cellular immunity (macroghages, natural killer cells, T lymphocyte) and humoral (antibodies). Although antibodies represent the essential means for diagnosing toxoplasmosis in humans, but the cellular immunity represent the key factor of the host's immune reaction in the event of attack by *Toxoplasma gondii* [66].

Neutrophils facilitate establishment of parasite- host interaction, they may ease transmission of parasite by support an effective immune response which helps in survival of the host and establishment of

infection. Also these cells play important role for the host by their effective action in destroyers of the parasites [106].

The NBT test used for studying the role of phagocytosis in toxoplasmosis infection, its elevated levels reflected an effectiveness of the innate immunity, as well as this test could explain some immunopathological aspects of this disease which may help in diagnosis of patient with *T.gondii* infection [107].

The Nitroblue tetrazolium stain (NBT) is a yellow crystal of salt, used particularly for oxidation of compound found on granule in neutrophils. In Nitroblue tetrazolium test, neutrophils ingest the yellow dye (NBT) and in the presence of reactive oxygen species, the (NBT) yellow colored compound is converted to (formazan) which is purple-blue compound [108]. This test involves incubation of heparinized blood samples with buffered NBT solution. After smear preparation and staining, the smear were examined under the microscope to find out the percentage of active neutrophils that exhibiting intracytoplasmic deposits of NBT-formazan. This percentage is usually increased in bacterial infections [109].

1.12 Hematological parameters:

1.12.1 Platelet:

Platelets or "thrombocytes" are fragments of large bone marrow cells called megakaryocytes that assist in blood clotting by clump together (aggregate) during normal blood clotting [110]. Platelets are small sized (2–3 μm) in diameter, disk shaped cell with no nucleus [111]. The lifespan of platelet is 5 - 9 days. Excessive bleeding occurs if the level of platelets is too low. However, blood clots form (thrombosis) if the level of platelets is too high. During pregnancy the changes in platelet levels are frequent. Mostly the platelet count decreased and this is because of haemodilution

which is become more frequent when the pregnant women enter in the third trimester [112].

A previous study on the contribution of human platelets in Toxoplasmosis by Zaki, M.M. in 2011 shows that " *T. gondii* tachyzoites could activate human platelets during primary infection and reactivated toxoplasmosis; this was supported by the potent cytotoxic activity exerted by human platelets against tachyzoites" [113].

1.12.2 White blood cells (WBC):

White Blood Cells or leukocytes (WBCs), its name comes from the physical appearance of a blood sample after separation by centrifuge. White cells are found in white layer of nucleated cells between the blood plasma and sediment red blood cells . The WBC cells of the immune system involved in protection the body against both foreign invader and infectious disease. All WBC cells are produced and derived from hematopoietic stem cell in the bone marrow. Leukocytes are found throughout the body, including the blood and lymphatic system [114].

Changes in the number of WBCs in the blood may indicate the presence of disease. The normal vale of WBC ($4x10^9$ and $1.1x10^{10}$) cells/L of blood, and it constitute about 1% of total blood in a healthy individual. There is five different types of leukocytes exist, in which the first group is called granulocytes (neutrophils, eosinophils and basophils) and the second group called agranulocytes (monocytes and lymphocytes) [115].

Leukocytosis occur when leukocytes increase in number above upper limits and (leucopenia) occurs when leukocyte decrease below the lower limit. Sometimes, leukocytosis represents a sign of more serious

primary bone marrow disease as leukemias or myeloproliferative disorders [116].

1.12.3 Hemoglobin (Hb):

Hemoglobin is the major oxygen carrying component in blood and is essential to the survival of most multicellular animals. Hemoglobin acts as transporter of other gases; it carries about 10% of carbon dioxide by binding of CO2 with the globin protein, also hemoglobin carries a nitric oxide molecule which is one of the important regulatory molecules [117].The major Hb in normal adults is hemoglobin A, a tetramer consisting of one pair of alpha chains and one pair of beta chains [118]. It is the main protein constituent of RBCs (92% of dry weight). In anemia either the number of red blood cells is insufficient to meet the physiological demand of the body or when Hb is below a certain limit which (13 g/dL)for men and below (12 g/dL) for women[119] , and this is vary by age ,sex , the state of pregnancy , etc.

Reduction in Hb concentration in women infected with toxoplasmosis may be attributed to multiplication of parasite inside the host body cells [120]. Also may be due to degradation of red blood cells which may cause anemia. Moreover during pregnancy the plasma undergoes relatively slight increase compared with RBC which may result in heamodilution which called (physiological anemia of pregnancy) [121].

1.12.4 Packed cell volume (PCV)

The packed cell volume is known as hematocrit , which is the volume of red blood cells in the whole blood. The reference value is about 45% for men and 40% for women [122]. The value of hematocrit is more than hemoglobin value by approximately 3 times. The PCV is an important part in complete blood picture results, in addition to, WBC count, platelet count and hemoglobin concentration [123].

The PCV which is the red blood cell volume percentage, helpful in determination of oxygen transport function; since the function of RBC is to transfer oxygen gas to all tissues of the body. So individual with high PCV level indicate the ability of the blood to transfer oxygen, while low level of PCV may be a sign of anemia .Therefore its act as a marker of health condition; and tests of hematocrit levels are often done in diagnosis of such conditions like, anemia [123].

1.13 Aim of the study:

1- To evaluate the relationship between hormonal levels (progesterone, estradiol, Follicle stimulating hormone, Luteinizing hormone and prolactin) and acute *T. gondii* infection in female patients. Focusing on hormonal changes before and after spiramycin treatment.

2- To determine the changes of some hematological parameters in female patients with acute *T.gondii* infection (Hemoglobin, Platelets, white blood cell, packed cell volume) using sysmex machine before and after spiramycin treatment.

3- To study the phagocytic activity of neutrophils by using Nitro-blue tetrazolium stain (NBT) in female patients infected with acute toxoplasmosis before and after spiramycin treatment.

Chapter Two

Materials and Methods

2.1 Materials:

2.1.1 Instruments: The instruments that used in this work with their manufactures as shown in table (2-1).

Table (2-1): The Instruments with their suppliers:

	Instrument	Company	Origin
1	Centrifuge	Hettich EBA20	germany
2	Deep freeze	REVCO	USA
3	COBAS®4000 e411	roche	Germany
4	Refrigerator	Kokusan	Japan
5	Sysmex	Sysmex H18	France
6	Refrigerator	arctiko	Denmark
7	Microscope	olympus	Japan
8	Incubator	memmert	Germany

2.1.2 Equipments :

The equipments that used in this study and their suppliers.

Table (2-2) The equipments with their suppliers:

	Equipment	Company	Origin
1	EDTA tubes	Afco	Jordan
2	Plain tube	Afco	Jordan
3	Eppendroff	Lp italiana spa	Italy
4	Micropipettes	Slamed	Germany
5	Disposable Pipette tips	AfCO	Jordan
6	Surgical gloves	bilim	turkey
7	Disposable Syringe	unlock	UK

2.1.3 Laboratory kits:

Laboratory kits that used in this work with their manufactures are listed in table (2-3)

Table (2-3): Laboratory ECLIA kits that used in this work:

	Kits of ECLIA	**company**	**origin**
1	Anti *Toxo* IgM	Roche	Germany
2	Estrogen	Roche	Germany
3	Progesterone	Roche	Germany
4	Prolactin	Roche	Germany
5	LH	Roche	Germany
6	FSH	Roche	Germany

2.1.4 Drugs & chemicals :

Drugs and chemicals that used in this study with their suppliers.

Table (2-4): chemicals and drugs with their suppliers:

	Drugs & chemicals	**Manufactures**
1	Spiramycin tablet 3 M.I.U	Sanofi-Aventis , France
2	Nitro-blue tetrazolium stain	BDH biochemical company , England
3	Phosphate-buffered saline solution , pH 7 .2	MP Biomedicals, USA
4	Leishman’s stain	MP Biomedicals, USA

2.2 Clinical studies :

2.2.1 Subjects :

This study was performed on 426 pregnant and non pregnant women with or without history of obstetric problems attending alkut Maternity Hospital and Al Kut medical laboratory in Wasit province between September 2014 to May 2015. Their ages ranged from 18 to 40 years, pregnant women were in the 1st to 3rd initial months of their pregnancy period.

All women were examined by using Cobas®4000 e 411 analyzer. Out of 426 women, (75) 15% appeared to be IgM positive for Toxoplasmosis and (351) 85% were IgM negative. 64 out of 75 women (42 pregnant and 22 non pregnant) that proved to have acute toxoplasmosis infection were treated with spiramycin, one tablet (3 million I.U) twice daily for two months.

The control group were 24 apparently healthy women (11 pregnant and 13 non pregnant), were free from signs and symptoms of other disease such as diabetes mellitus, hypertension, obstetric problems and were seronegative IgM for Toxoplasma infection. An information sheets were prepared and designed according to a questionnaire before collection of the samples (Appendix).

2.2.2 Blood sampling:

Six ml of venous blood sample was withdrawn from each woman by using disposable syringes. One ml of blood was placed in EDTA tube for hematological test which involve determination of blood parameters (HB, PCV, WBC, Platelets), and another one ml of blood for NBT stain to determine phagocytic activity. The remaining four ml of blood was placed in plain tubes and kept at room temperature for clotting, then the serum was separated by centrifuge at 3000 round per minute (rpm) for about 10 minutes.

Then the serum transferred immediately to another special tubes which were formulated specifically for Cobas®4000 e411 analyzer for screening of anti-*Toxoplasma* IgM.

After getting the results of IgM, the remaining serum for those who were positive IgM were transferred to eppendrof tubes by using micropipette and stored at -20 C for subsequent hormonal analysis.

After two months of spiramycin treatment, another blood sample (6 ml) was collected from each seropositive woman. Each sample was divided into 2 portions (2ml) for hematological analysis and phagocytic activity. The remaining 4ml are centrifuged and the serums were kept at -20 C for subsequent hormonal analysis.

2.2.3 Experimental Design :

The design of the study is illustrated in the following diagram.

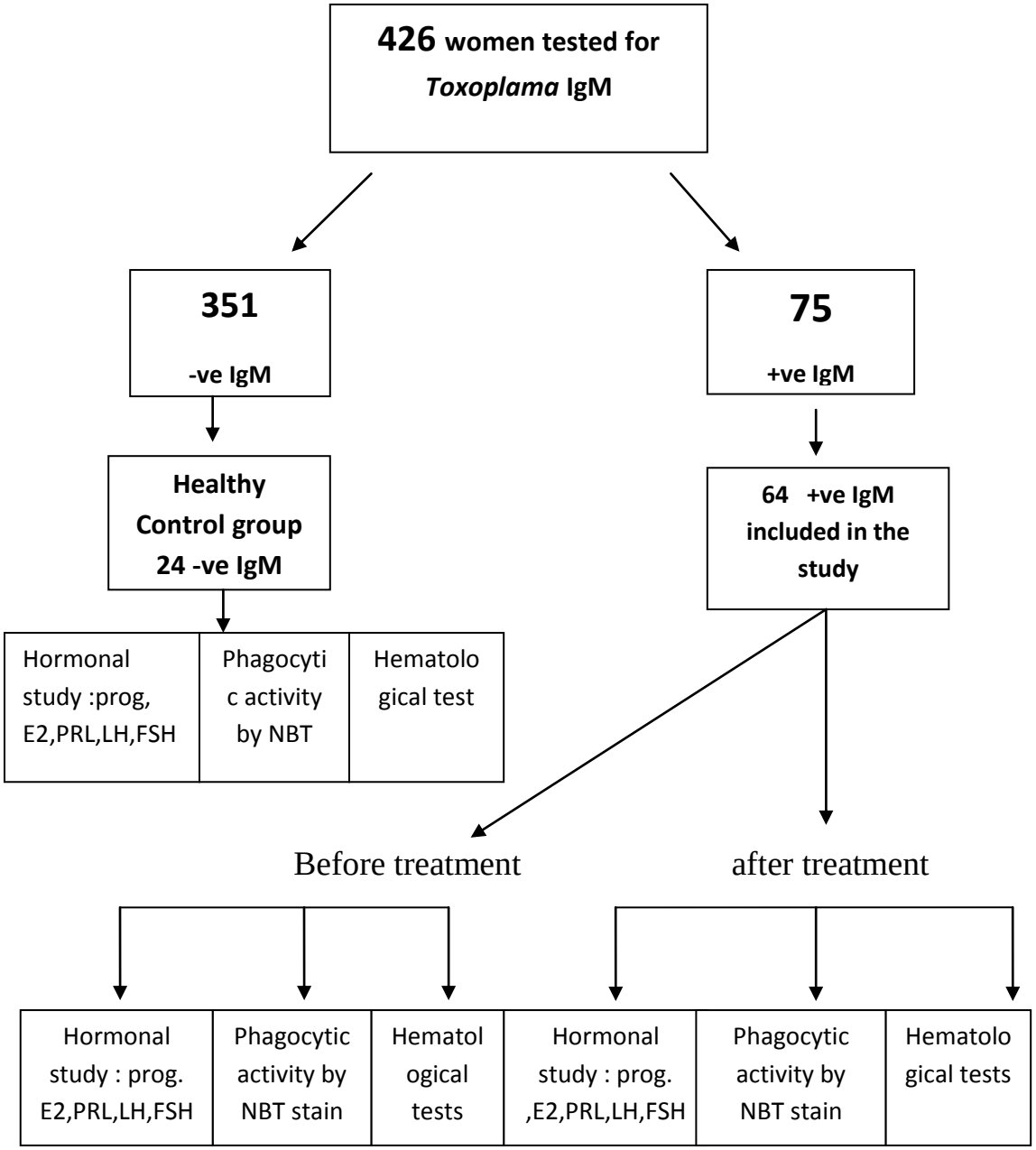

Diagram (2-1): Experimental plan design

2.3 Detection of (IgM) specific anti-*Toxoplasma gondii* antibody via using Electrochemiluminescence (ECL) technique:

Kits for detection of IgM antibodies against *T. gondii* antigens in human serum (Roche Diagnostics GmbH, Mannhein, Germany) was used according to company`s information.

Anti-*Toxoplasma* IgM detection was used to evaluate a patients serologic status to *T.gondii* infection. The sera were examined for anti-*Toxoplasma* IgM antibodies by using an automated analyzer Cobas® 4000 platform the (Cobas e 411 analyzer*)* which is a fully automated machine and immunoassay analysis controlled by software system. The software performed automatically transmission of information to and from the analyzer, results assessment, documentation and quality control.

Cobas e 411 is based on electrochemilumenisce (ECL) technique which is "Electro" refers to electrical stimulation, "Chem" point to a chemical reaction "Luminescence" refers to light production[65]. This technology provides superior analytical performance and increased sensitivity which means that it can detect extremely low level of antigen, as well as slight changes in antigen level [65].

Figure (2-1): Cobas e 411 analyzer

2.3.1 The principle of the assay :- (124)

This assay is taken about 18 minutes.

1- First incubation: involve dilution of sample (10) µL in a ratio (1: 20) automatically with Universal Diluents. After that added *T.gondii*-Ag which is labeled with a ruthenium complex*. Followed by reaction of (IgM) antibodies with the ruthenium-labeled *T. gondii*.

2- Second incubation: included adding both Biotinylated monoclonal h-IgM-particular antibodies with streptavidin-covered microparticles to form complex. Then this complex binds to the solid phase through reaction between streptavidin and biotin.

3- Inside the measuring cell, aspiration of the reaction mixture occurred where the microparticles were attracted magnetically on top of electrode surface; then using ProCell/ProCell M to eliminate unbound substances. Use of passing voltage through the electrode followed by chemiluminescent emission that measured by using a photomultiplier.

*Tris(2,2'-bipyridyl)ruthenium(II)-complex (Ru(bpy))

4- The software responsible for determination of the results automatically by comparing between the emission signals of electrochemi-luminescence and value of the cutoff that determine by calibration.

2.3.2 Assay reagents :

The pack of the reagents included three rack pack labeled with (M, R1, and R2)

- First bottle (6.5 ml) labeled with (**M**) that covered with transparent cup contain Streptavidin-coated microparticles (0.72 mg/mL) with preservative.
- Second bottle labeled with (R1**)** that enclosed with gray cover contain (9 mL) of Toxoplasma-antigen labeled with ruthenium complex > 1 mg/L (Toxoplasma-Ag~Ru[bpy]); 2-morpholino-ethane sulfonic acid (MES) buffer (50 mmol/L) , (pH 6)and preservative.
- Third bottle sized (9 mL) labeled with (**R2)** have (black cap) contain Biotinylated monoclonal anti-h-IgM antibody (mouse) > 500 µg/L (Anti-h-IgM-Ab~biotin); [4 -(2-hydroxyethyl)-piperazine]-ethane sulfonic acid (HEPES) buffer 50 mmol/L , pH 7.2 and preservative.

TOXIGM Calibration 1: container with the (white cover) -ve calibrator 1, include two bottles (0.67ml), each one contain Human serum that is negative for anti-*Toxo* IgM and preservative.

TOXIGM Calibration 2: container with the (black cover) +ve calibrator 2, include two bottles (0.67 mL) each one contain: Anti-Toxo IgM (human) (130 U/mL) in human serum and also contain preservative.

2.3.3 Assay procedure:

The procedure of detection of anti-*Toxoplasma* IgM according to manufacturer (Roche Diagnostics GmbH, Mannhein, Germany).

1. Wash solution system was prepared by adding a 30 ml of wash system to 3L of deionized water.
2. The procell and the clean cell were placed in their special places in the machine.
3. Both assay cup and tips were put in their places in the machine
4. Then the (start) bottom was pressed.
5. The user name and password were entered.
6. Maintenance process and system reset were performed.
7. The kits were placed in the reagent disk.
8. reagents scan were performed
9. The calibrator was placed in the sample disk(the capacity 30 test)
10. The barcode special for each test was read through (barcode scan).
11. The calibration processes were performed.
12. Reagent sample was adding to control and it has been read by a special barcode by using a (control barcode).The control process was conducted and the results were within the SD1 level and close to the mean which indicates the accuracy of the results according to the leveling chart.
13. Tests were conducted on the samples.

2.4 Estimation of serum hormones (Estradiol , Progesterone , Prolactin , Follicle stimulating hormone and Luteinizing hormone) levels by ECLIA technique :

2.4.1 Estradiol:

The estradiol II Immunoassay test kit was used. Cobas e411 analyzer used for the quantitative determination of estradiol (E_2) concentration in human sera.

2.4.1.1 The Principle of the assay: [125]

Test principle is based on competition principle and a total assay take about 18 minutes.

1- First incubation: include incubation both (35 µL) of the sample with an estradiol-specific biotinylated antibody for immunocomplex production, the analyte concentration in the sample determine the complex amount.

2- Second incubation: in this step the empty positions of the biotinylated antibodies were occupied after addition of an estradiol derivative labeled with both (ruthenium complex) and (streptavidin-coated microparticles) , then whole complex bind to the solid phase.

3- Inside measuring cell aspiration of the mixture wherever the micro particles are attracted magnetically on top of electrode surface. Then using ProCell/ProCell M to eliminate unbound substances. Use of passing voltage through the electrode followed by chemiluminescent radiation that detected by photomultiplier.

4- Calibration curve was used for results determination.

2.4.1.2 Assay reagents:

The rackpack of reagents is labeled with E2 II.

- The first bottle labeled **M** having (transparent top) size 6.5 mL of Streptavidin-coated microparticles, contain: preservative and (0.72 mg/mL) of Streptavidin-coated microparticles.
- The second bottle (8 mL) with the (gray cap) labeled with **R1** , contain : Biotinylated polyclonal anti-estradiol antibody (rabbit) 45 ng/mL; 130 ng/mL of Mesterolone ; (50 mmol/L) of MES buffer, pH 6.0 and preservative
- The third bottle with **R2** label (8ml) of Estradiol-peptide~Ru(bpy), covered with black cap

  Contains: Estradiol derivative with ruthenium complex label (2.75ng/ml); (50 mmol/L) of MES buffer, pH 6.0 and preservative.

2.4.2 Progesterone:

Progesrton II hormone test kit was used for quantitative determination of progesterone in human serum. The electrochemiluminescence immunoassay "ECLIA" is used on Cobas e411 immunoassay analyzer.

2.4.2 .1 The principle of the assay:

The test based on competition principle. The duration time take a (18 minutes) for the total test.

1- The first incubation: include incubation of (30 µL) sample - in the presence of a progesterone derivative labeled with ruthenium complex[*)] and a biotinylated monoclonal progesterone-specific

antibody with Danazol for progesterone releasing. Progesterone from the sample competes with the labeled progesterone derivative for the AB binding site.

2- The second incubation: include binding of the complex to the solid phase (through streptavidin and biotin interaction) after addition of streptavidin-coated microparticles. The amount of progesterone in the sample is inversely proportional to the amount of the labeled progesterone derivative.

3- Inside measuring cell; aspiration of the mixture wherever the micro particles are attracted magnetically on top of electrode surface; Then ProCell/ProCell M were used to eliminate Unbound substances. Next passing of a voltage through electrode followed by chemiluminescent radiation that detected by a photomultiplier.

4- Calibration curve used to determine the results.

*) Tris (2, 2'-bipyridyl) ruthenium (II)-complex (Ru(bpy))

2.4.2.2 Assay reagents:

The rackpack of this reagent is labeled as PROG II.

- Include bottle (6.5 mL) labeled with **M** enclosed with (transparent cover) Streptavidin-coated microparticles contain: preservative and Streptavidin-coated microparticles (0.72 mg/mL).
- Include bottle of 10 ml Anti-progesterone-Ab~biotin, enclosed with (gray cover) label with **R1**, contain: Biotinylated monoclonal anti-progesterone antibody (mouse) (0.15 mg/L), pH 7.0; preservative, phosphate buffer 25 mmol/L.

- Progesterone-peptide~Ru(bpy), 1 bottle (8 mL) labeled with **R2 ,** enclosed by (black cover) contain : Progesterone (of vegetable origin) attached to a synthetic peptide labeled with ruthenium complex, pH 7.0; preservative ,10 ng/mL; phosphate buffer 25 mmol/L.

2.4.3 Follicle Stimulating Hormone (FSH)

The FSH hormone test kit was used for the quantitative determination of follicle-stimulating hormone of human sera. Using of Electrochemiluminescence immunoassay technique "ECLIA" on Cobas e411 immunoassay analyzers, according to manufacturer's instructions.

2.4.3.1 The Principle of the assay:

This test based on sandwich principle. This test is takes about 18 minutes.

1- First incubation: involve formation of a sandwich complex by reaction of (40 µL) of sample (a biotinylated monoclonal FSH-specific antibody) with a monoclonal FSH-specific AB labeled with a ruthenium complex*.

2- Second incubation: involve binding of the whole complex to the solid phase, after adding of streptavidin-coated microparticles.

Inside measuring cell; aspiration of the mixture wherever the micro particles are attracted magnetically on top of electrode surface; then using ProCell/ProCell M to eliminate unbound substances. Use of

*(Tris(2,2'-bipyridyl)ruthenium(II)-complex (Ru(bpy))

passing voltage through the electrode followed by release of chemiluminescent that can be detected by a photomultiplier.

3- Calibration curve used to determine the final results.

2.4.3.2 Assay Reagents:

The rackpack of the reagent is labeled as FSH.

- The first bottle enclosed by transparent cover and label with **M** (6.5 mL) of Streptavidin-coated microparticles, contain: Streptavidin-coated microparticles (0.72 mg/mL) with preservative.
- The second bottle of (10ml) Anti-FSH-Ab~biotin, labeled with **R1** and enclosed by (gray cover), contain: 0.5 mg/L of Biotinylated monoclonal anti-FSH antibody (mouse), pH 6.0; preservative, (50 mmol/L) MES buffer.
- The third bottle of (10 mL) Anti-FSH-Ab~Ru(bpy) labeled with **R2** sealed by (black cover) contain : (0.8 mg/L) of Monoclonal anti-FSH antibody (mouse) labeled with ruthenium complex, pH 6.0; preservative ,MES buffer 50 mmol/L.

2.4.4 Luteinizing hormone (LH):

Luteinizing hormone Immunoassay test kit was used for the quantitative determination of luteinizing hormone in human serum. The electrochemiluminescence immunoassay "ECLIA" is used on Cobas e411 immunoassay analyzers.

2.4.4.1 The principle of the assay:

Based on sandwich principle. Time duration for this assay is take s about 18 minutes.

1. The first incubation: sandwich complex formation result by reaction (20 µL) of sample, a monoclonal LH-specific antibody labeled with a ruthenium complex with a biotinylated monoclonal LH-specific antibody.
2. The second incubation: in this step the whole complex becomes bound to the solid phase by (streptavidin and biotin) reaction and after addition of streptavidin-coated microparticles.

5- Aspiration of the mixture occurred inside measuring cell the in which the microparticles are caught magnetically on top of the electrode surface. Then using ProCell/ProCell M to eliminate unbound substances. Next passing voltage through the electrode followed by chemiluminescent radiation that can be detected by photomultiplier.

6- Calibration curve used to determine the final results.

2.4.4.2 Assay reagents:

The reagent rack pack is labeled as LH

- One bottle of Streptavidin-coated microparticles (6.5ml) labeled with **M** and enclosed by transparent lid include: Streptavidin-coated microparticles, which contain: 0.72 mg/mL of Streptavidin-coated microparticles plus preservative.
- Bottle of (10 mL) Anti-LH-Ab~biotin, labeled with **R1** covered with (gray lid) contain: 2.0 mg/L of Biotinylated monoclonal

anti-LH antibody (mouse); pH 8.0; preservative, TRIS buffer 50 mmol/L.

- Bottle of (10ml) Anti-LH-Ab~Ru(bpy), labeled with **R2** covered (black lid) include contain : Monoclonal anti-LH antibody (mouse) labeled with ruthenium complex 0.3 mg/L; pH 8.0; preservative , TRIS buffer 50 mmol/L.

2.4.5 Prolactin:

Prolactin test kit was used for quantitative determination of prolactin in human serum. The electrochemiluminescence immunoassay "ECLIA" was used on cobas e411 immunoassay analyzers.

2.4.5.1 The principle of the assay:

This test based on sandwich principle. Time duration takes about 18 minutes.

1- The first incubation: involves the formation of the first complex by adding about (10 µL) of the sample with a biotinylated monoclonal prolactin specific AB.

2- Followed by second incubation: which involves the formation of sandwich complex by adding streptavidin-coated microparticles and a monoclonal prolactin-specific AB which have ruthenium complex label *) , the whole complex will be tied to the solid phase through reaction between streptavidin and biotin.

3- Aspiration of the mixture inside measuring cell in which microparticles are caught magnetically caught on top of electrode surface.

*(Tris(2,2'-bipyridyl)ruthenium(II)-complex (Ru(bpy))

Then using ProCell/ProCell M to eliminate unbound substances. After that passing of voltage through the electrode followed by chemiluminescent radiation which is can be detected by a photomultiplier.

4- Calibration curve was used to determine the final results.

2.4.5.2 Assay reagents:

The rack pack of the reagents is labeled as PRL II.

- The first bottle (6.5ml) labeled with **M** covered with transparent lid, contian Streptavidin-coated microparticles, which include ; 0.72 mg/mL of Streptavidin-coated microparticles with preservative.
- The second bottle (10 mL) of Anti-prolactin-Ab~biotin labeled with **R1**enclosed by gray cover, contain: (0.7 mg/L) of Biotinylated monoclonal anti-prolactin antibody (mouse); pH 7.0; preservative, phosphate buffer 50 mmol/L.
- The third bottle (10 mL) of Anti-prolactin-Ab~Ru(bpy) labeled with **R2** covered with black lid, contain : Monoclonal anti-prolactin AB (from mouse) labeled with ruthenium complex 0.35 mg/L; pH 7.0; preservative ,phosphate buffer 50 mmol/L.

2.4.5.3 Assay procedure:

For all hormones they have the same working procedure (according to Roche Diagnostics GmbH, Mannhein, Germany) which include:

1. The serum was separated from blood by using centrifuge apparatus.
2. The serum (200Mg) was put in special cups of the apparatus.

3. Then the cups were place in special rack of the apparatus with the right numbers written on the screen
4. The rack of the kits was placed inside the reagent disk.
5. Patient data was written on the screen (patient ID, name, sample number) then press enter.
6. Next the required investigations were selected (E2 , PROG,FSH ,LH ,PRL) for each sample in the test rack
7. After ending, run was pressed to start device working.

2.5 Estimation of Phagocytic activity of neutrophils by using nitro-blue tetrazolium stain (NBT):

2.5.1 The Principle of the test:

A yellow crystal water soluble nitro-blue tetrazolium (NBT) dye was used for estimation the phagocytic activity of neutrophils. A positive result obtained when the active neutrophils ingest the NBT dye and convert the yellow colored of NBT to purple –blue formazan compound in the presence of reactive oxygen species [(126)]. This test can be done within 1 hour duration for specimen collection, Park method [(127)] was used with some modification [(128)].

The NBT test is a simple method that helpful in the diagnosis as well as management of infection. The percentage of neutrophiles that contain purple blue deposits (formazan) represents the phagocytic activity of the neutrophils[(108)].

2.5.2 Reagents:

1. Solution of 0.2% NBT was prepared by dissolve nitro-blue tetrazolium stain (200 mg) in about (100 ml) of phosphate buffered saline solution [129,130]. Followed by filtration of NBT solution into dark bottle, which is stable at room temperature for a several weeks and about year if frozen [131].
2. Solution of Phosphate buffered saline (pH 7.2).
3. Leishman's stain is used as a Counter stain.

2.5.3 Procedure:

1. About 1 ml of blood sample was withdrawn from the patient and placed in plain tubes, then heparin was added as anticoagulant (heparinized blood)
2. Two equal volumes of phosphate buffer saline and NBT solution were mixed in the test tube [132].
3. The heparinized blood were added about 0.1 ml to the mixture and all content were mixed.
4. The mixture was incubated at 37 Ċ for about (10 – 15) minutes then left at room temperature for further (10 – 15) minutes.
5. Next, the solution was mixed gently and blood smear was done, allow the smear to dry then stained by Leishman's stain as a counter stain[129].
6. The smears were examined under the microscope (low power)and 200 neutrophils were counted for patients group. The results were reported as the percentage of NBT positive neutrophils (that have large dark blue deposits) [127].

2.6 Estimation of Heamatological parameters (WBC, PCV, Platletes, Hb):

2.6.1 Principle:

The sysmex machine was used in this test which is hematology analyzer (fully automatic machine) using for measuring RBC count, hematocrit and impedance platelet count by direct current sheathed flow. Moreover; measuring leukocyte differential count, nucleated red blood cells (NRBC) count, reticulocyte count, and impedance platelet count by using of fluorescent flow cytometry with a semiconductor laser and fluorescent dyes. In addition; the differential leukocyte counts analyzed by using optical information, this machine detected three types of optical information; which include; forward-scattered light, side-scattered light and side fluorescence light. There is a separate channel called immature myeloid information (IMI) provides additional information of WBC on the presence of immature granulocytes, hematopoietic progenitor cells and blasts[(133)].

3.6.2 Procedure:

The hematological parameters were measured by Blood Coulter machine. A blood sample was withdrawn from the patient placed in a special tube container EDTA; solution used in this machine was three types; Isotonic diluent, lyse solution, cleaning agent. Data was entered with the name of the patient and sample number inside the device by the keyboard related to the computer unit built with the device. The results were printed out by special paper.

2.7 Statistical Analysis

Data were analyzed by using Statistical Analysis System (SAS) - 2012 to study the affect of different factors in different parameters which were used in this study. Least significant difference test (LSD) (p value < 0.05) was used to compare between means of different groups in this study [134].

Chapter Three

Results

3.1 The changes in the hormonal levels in pregnant women infected with acute Toxoplasmosis before and after spiramycin treatment:

The results in table (3-1) showed that the mean concentration of hormones (progesterone, estrogen, follicle stimulating hormone, prolactin and luteinizing hormone) in pregnant infected women with acute toxoplasmosis before and after spiramycin treatment.

Table (3-1): The mean concentration of the hormones in the pregnant infected women before and after treatment comparing with control group:

parameter	Groups of pregnant women n=43				
	Mean ± SE				
	Control healthy subject n=11	**Before treatment n=43**	**After treatment n= 43**	**LSD**	**P-value**
Estrogen (pg/ml)	392.31±31.80 B	518.67 ± 34.42 A	190.34 ± 19.02 C	105.65 **	0.0001
Progesterone (pg/ml)	25.68 ± 3.75 A	22.82 ± 1.88 A	22.67 ± 1.46 A	6.750 NS	0.715
FSH (mIu/ml)	0.251 ±0.053 B	0.735 ± 0.24 A	0.341 ± 0.102 B	0.406 **	0.01219
LH (mIu/ml)	0.088 ±0.017 A	0.123 ± 0.026 A	0.133 ± 0.019 A	0.0826 NS	0.610
Prolactin (ng/ml)	41.00 ± 4.58 A	35.26 ± 3.80 A	39.51 ± 2.45 A	12.284 NS	0.542

Data were expressed as mean± SD; n=number of patients; values with (A, B, C) superscript in the same group represent significance differences; ** represent highly significant $p<0.05$; NS represent Non-significant difference between groups.

The result of estrogen (E_2) revealed significant increased ($P<0.05$) in the serum level of before treatment group (518.67 ± 34.42 pg/ml) compared with healthy control (392.31± 31.80 pg/ml). While significant reduction ($p<0.05$) in after treated group (190.34 ± 19.02 pg/ml) when compared with control and before treated groups, (Figure 3-1).

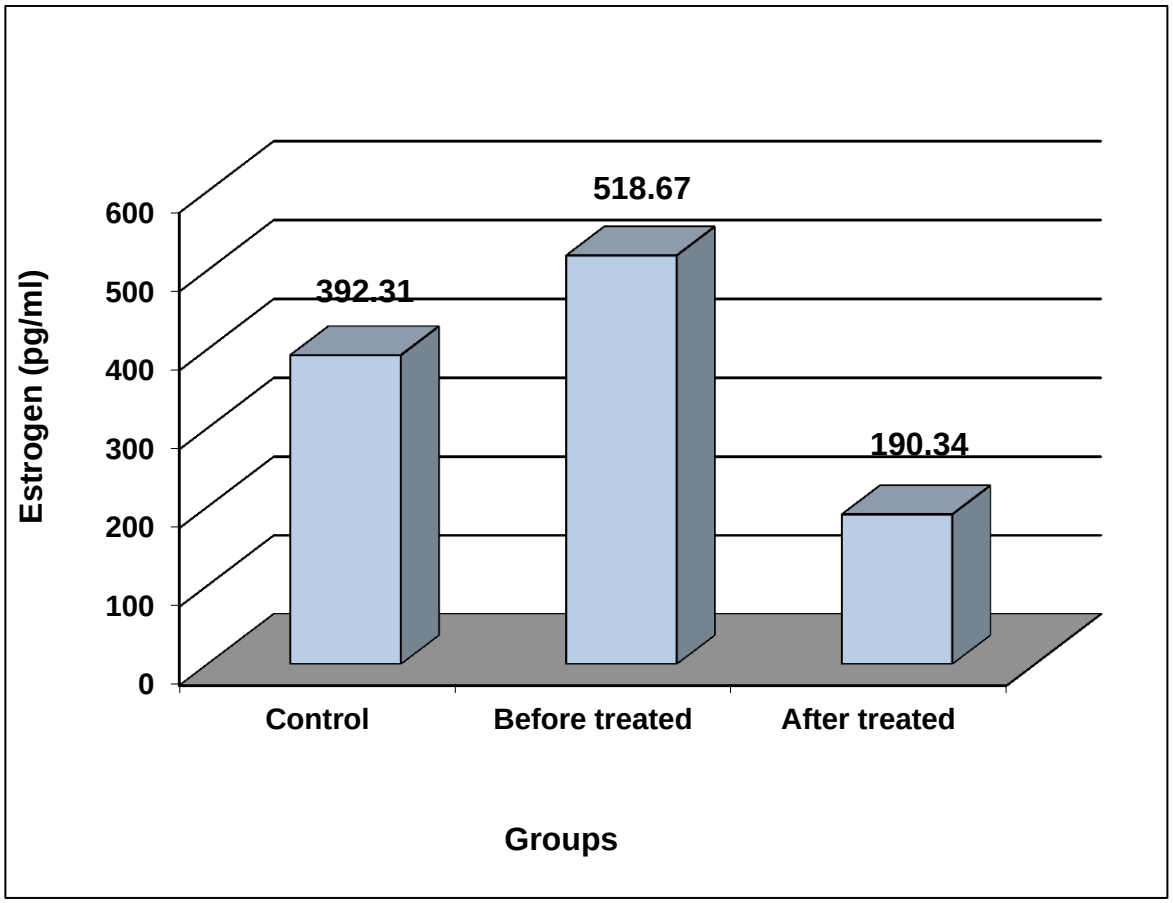

Figure (3-1): Mean values of serum Estrogen levels in pregnant infected with toxoplasmosis before and after treatment.

As motioned in table (3-1) the mean concentration of progesterone showed that there is no statistical difference between all groups (P>0.05), as show in Figure (3-2).

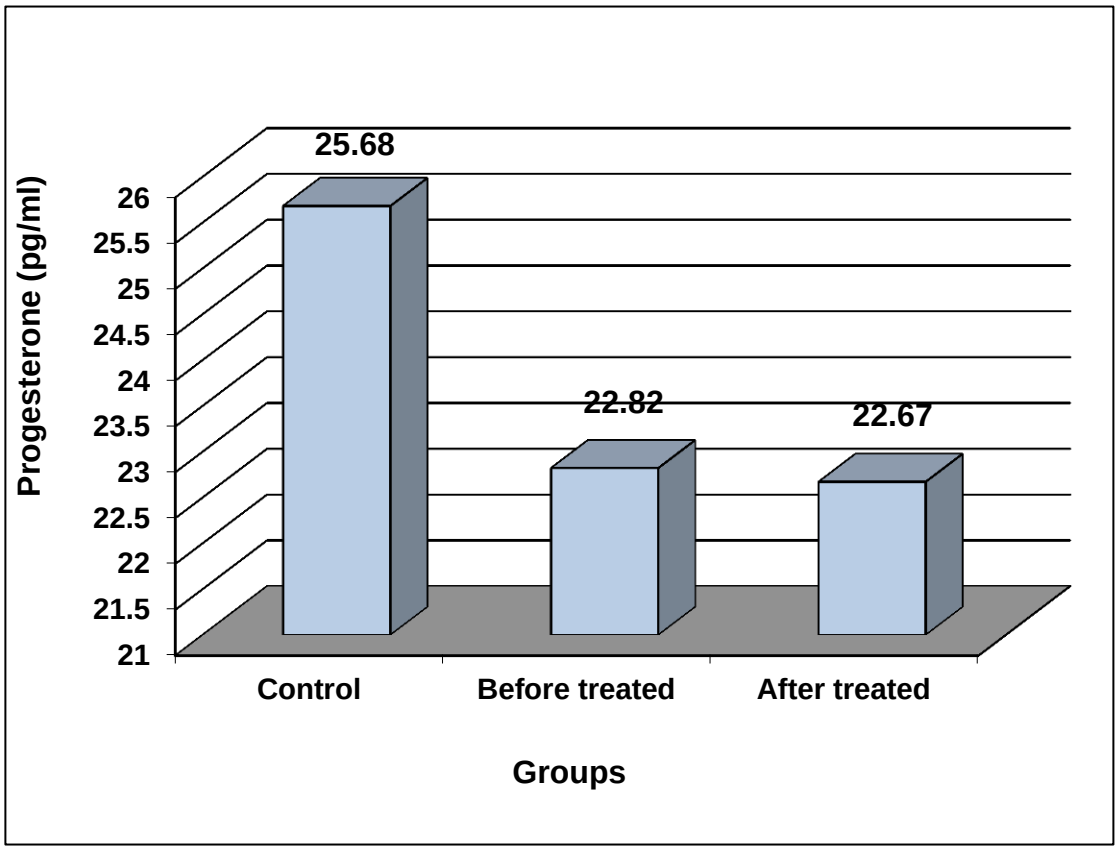

Figure (3-2): Mean values of serum progesterone levels in pregnant infected with toxoplasmosis before and after treatment.

In table (3-1) the result of follicle stimulating hormone (FSH) showed significant increased ($p<0.05$) before treatment (0.735 ± 0.24 µIu/ml) compared with healthy control (0.251 ± 0.053 µIu/ml) and after treatment (0.341 ± 0.102 µIu/ml). As showed below in figure (3-3).

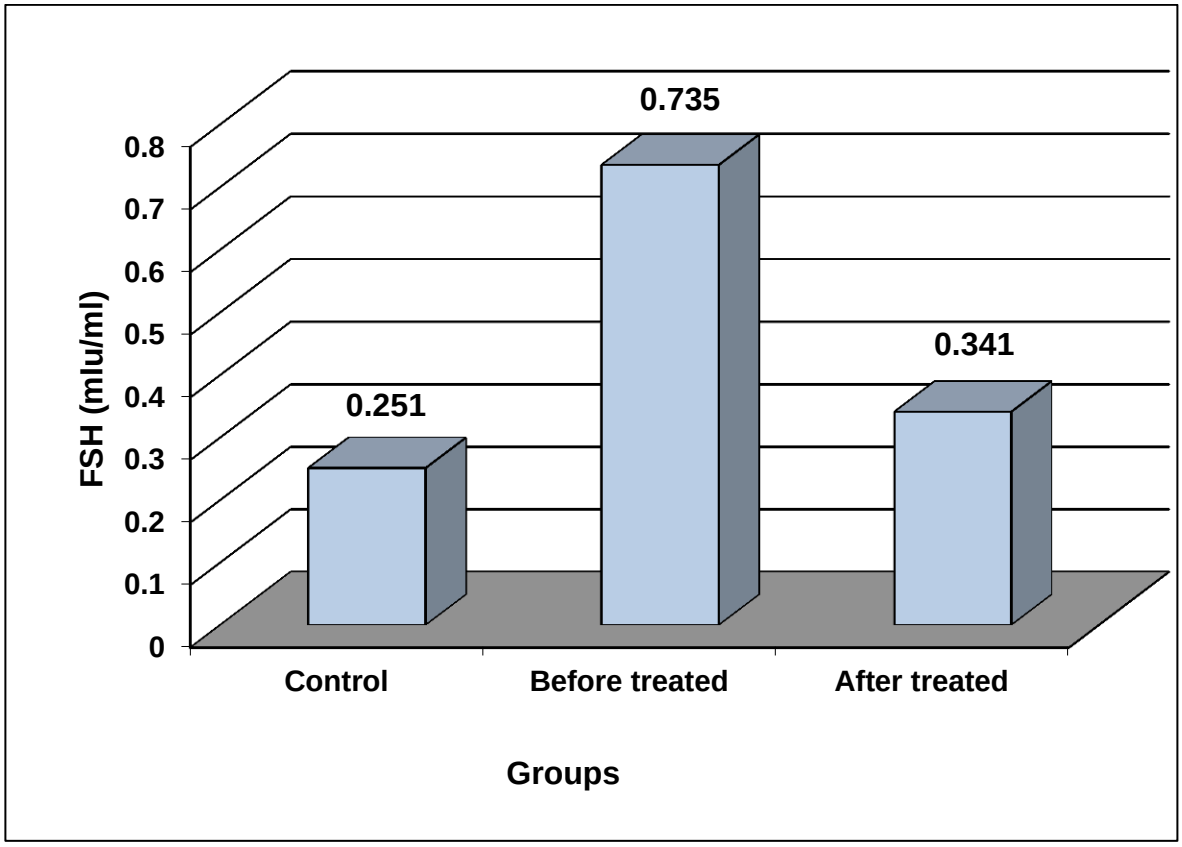

Figure (3-3): Mean values of serum FSH levels in pregnant infected with toxoplasmosis before and after treatment.

The data presented in table (3-1) revealed that there are no statically difference ($p>0.05$) in mean concretions of lutenizing hormone (LH) in toxoplasma patients before and after treatment when compared with healthy control groups.

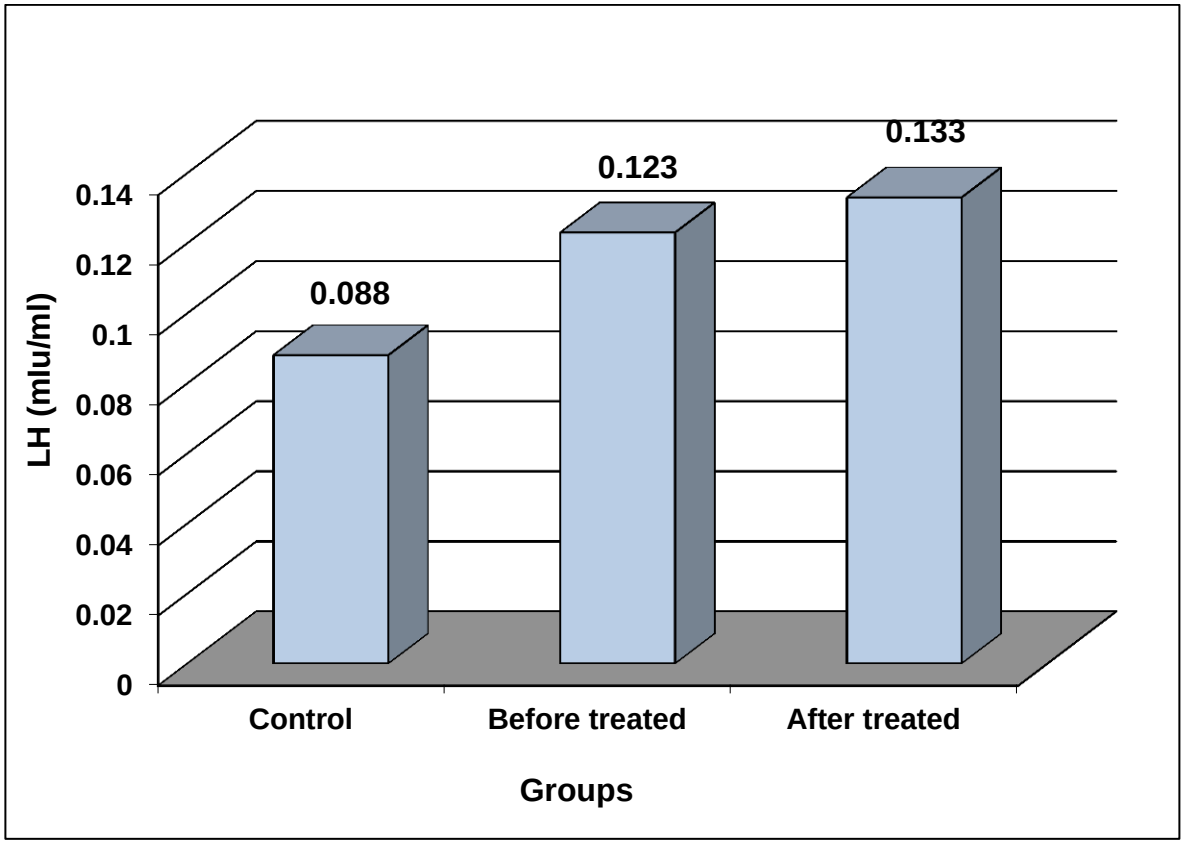

Figure (3-4): Mean values of serum LH levels in pregnant infected with toxoplasmosis before and after treatment.

Also the data in table (3-1) showed the mean level of prolactin not differ significantly ($p>0.05$) between the groups under study (pretreated, treated, control) as showed in figure (3-5).

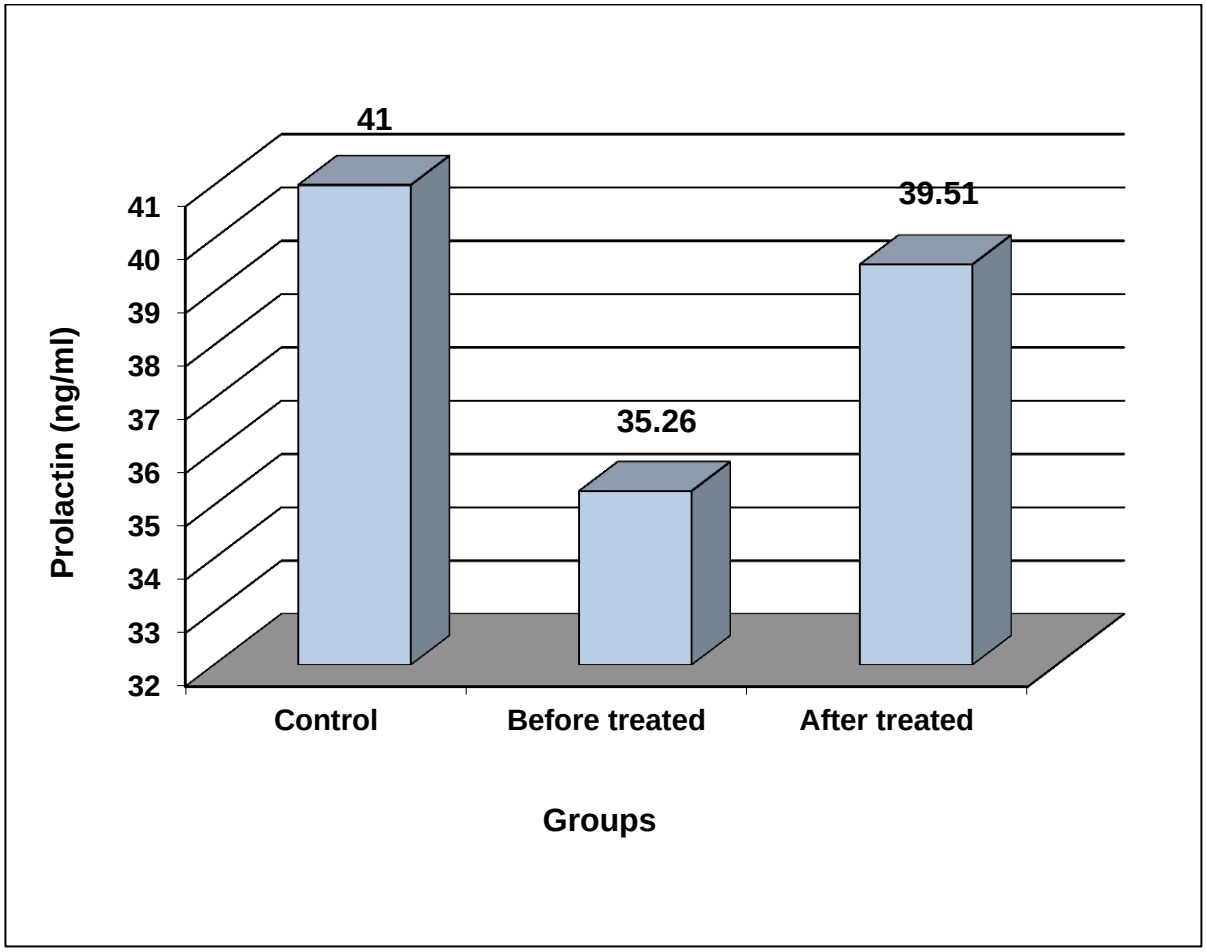

Figure (3-5): Mean values of serum prolactin levels in pregnant infected with toxoplasmosis before and after treatment.

3.2 The changes in the hormonal levels in non pregnant women infected with acute Toxoplasmosis before and after spiramycin treatment:

The results in table (3-2) showed that the mean concentration of hormones (progesterone, estrogen, follicle stimulating hormone, prolactin and luteinizing hormone) in non-pregnant infected women with acute toxoplasmosis before and after treatment

Table (3-2): The mean concentration of hormones in non pregnant infected women infected before and after treatment compared with control group:

parameters	Groups of non pregnant patients Mean ± SE				
	Control healthy subject n=13	**Before treatment n=22**	**After treatment n=22**	**LSD**	**p-value**
Estrogen (pg/ml)	51.26 ± 7.77 C	132.37± 9.94 A	80.45 ± 5.39 B	25.687 **	0.0001
Progesterone (pg/ml)	3.65 ± 1.80 A	2.78 ± 1.01 A	2.77 ± 0.35 A	2.943 NS	0.829
FSH (µIu/ml)	6.56 ± 1.33 AB	9.08 ± 3.19 A	2.84 ± 0.45 B	5.053 **	0.0128
LH (µIu/ml)	7.64 ± 2.54 AB	11.06 ± 3.95 A	2.56 ± 0.47 B	7.910 **	0.0153
Prolactin (ng/ml)	13.77 ± 1.09 A	12.36 ± 1.07 A	14.13 ± 0.92 A	3.196 NS	0.409

Data were expressed as mean± SD; n=number of patients; values with (A, B, C) superscript in the same group represent significance differences $p<0.05$; ** represent highly significant; NS represent Non-significant difference between groups.

The result of non pregnant infected women with acute toxoplasmosis showed significant increased ($p<0.05$) in the mean concentration of estrogen (E_2) before treatment (123.37 ± 9.94 pg/ml) comparing with control group (51.26 ±7.77 pg/ml) and after treated group (80.45± 5.39 pg/ml).

In table (3-2), the mean estrogen level also showed significant increased ($p<0.05$) in after treatment in comparing with control groups. As shown in figure (3-6).

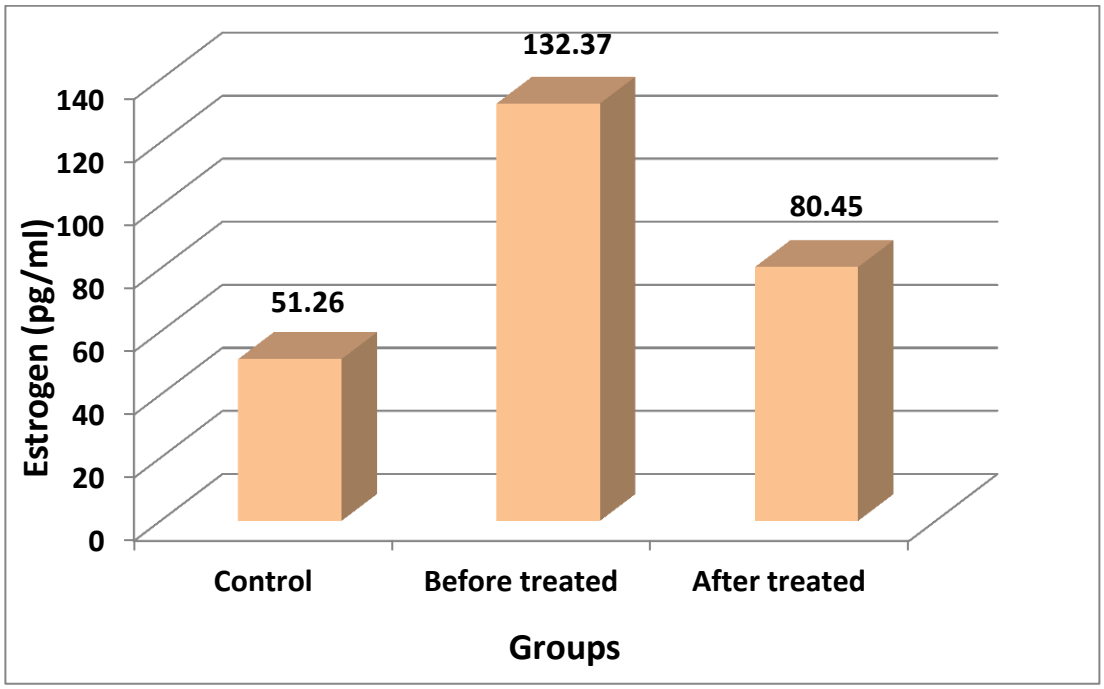

Figure (3-6): Mean values of serum Estrogen levels in non pregnant infected women with acute toxoplasmosis before and after treatment.

From Table (3-2) the result showed that the mean concentration of progesterone was not differ significantly between all groups of the present study (p>0.05). As below in figure (3-7).

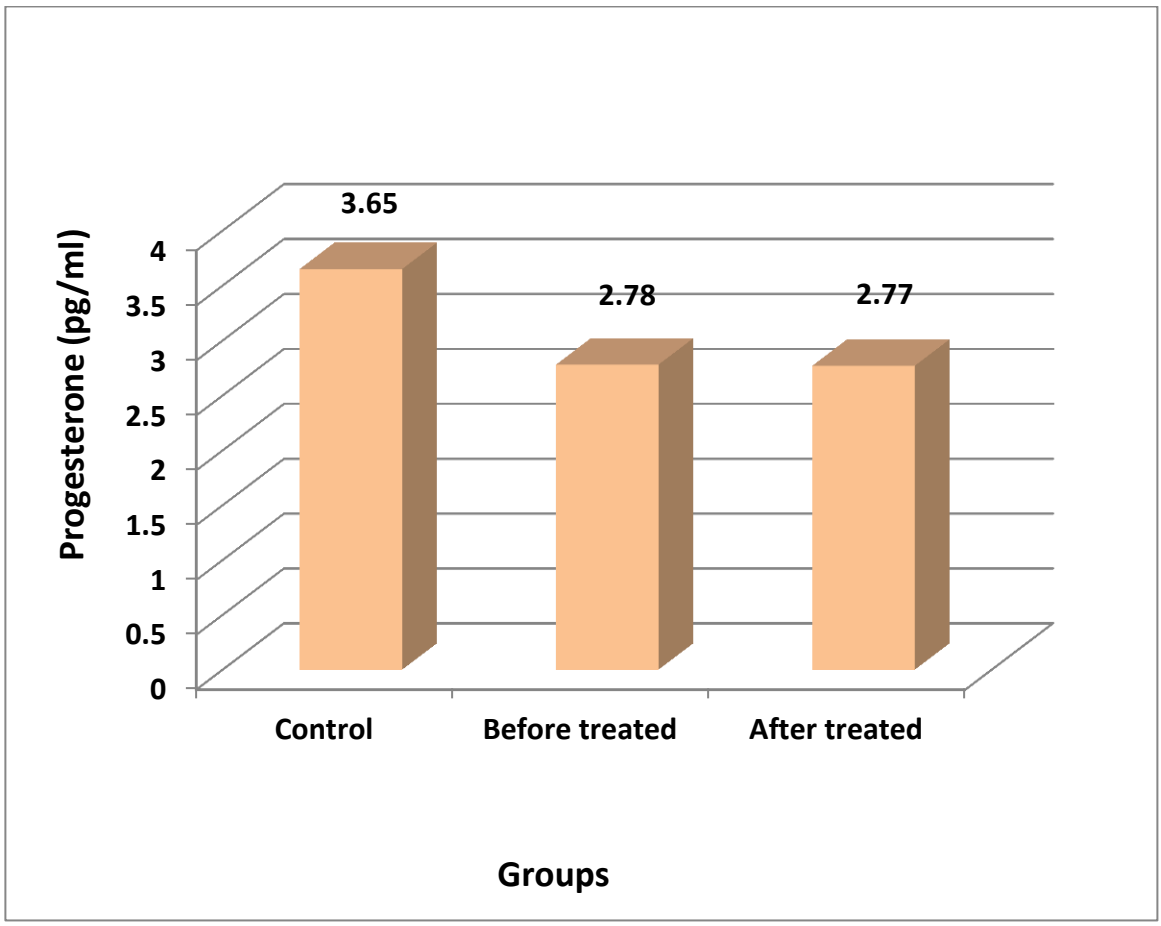

Figure (3-7): Mean values of serum progesterone levels in non pregnant infected women with acute toxoplasmosis before and after treatment.

Also the results in same table (3-2) showed significant increased in the mean level of FSH (p<0.05) in pretreatment group (9.08± 3.19 mIu/ml) compared to control group (6.56 ±1.33 mIu/ml). Whereas, significant decreased in post treated group (2.84 ± 0.45 mIu/ml) compared with pretreated and control. Figure (3-8).

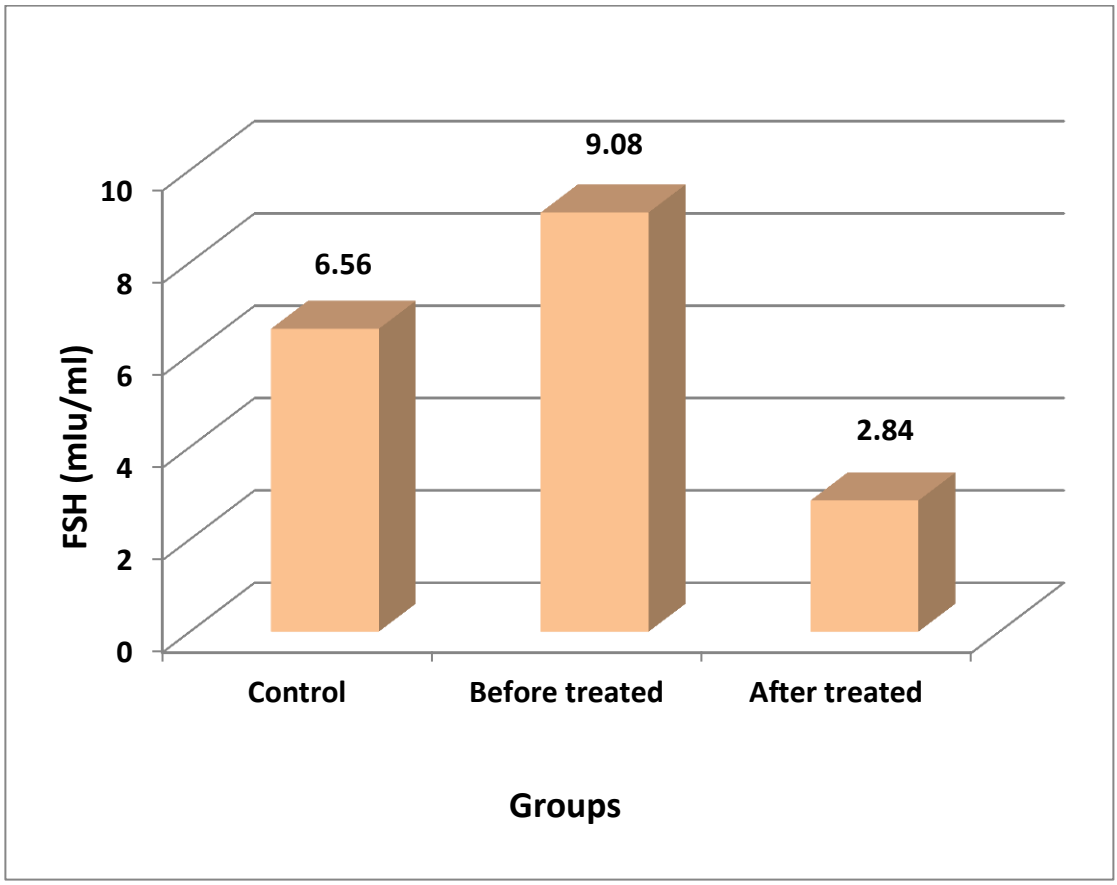

Figure (3-8): Mean values of serum FSH levels in non pregnant infected women with acute toxoplasmosis before and after treatment.

The mean level of LH was significant increased ($p<0.05$) in before treated group (11.06 ± 3.95mIu/ml) compared with control groups (7.64 ± 2.54 mIu/ml), and significant decrease ($p<0.05$) in after treated (2.56 ± 0.47 mIu/ml) compared with both before treated and control groups, table (3-2).

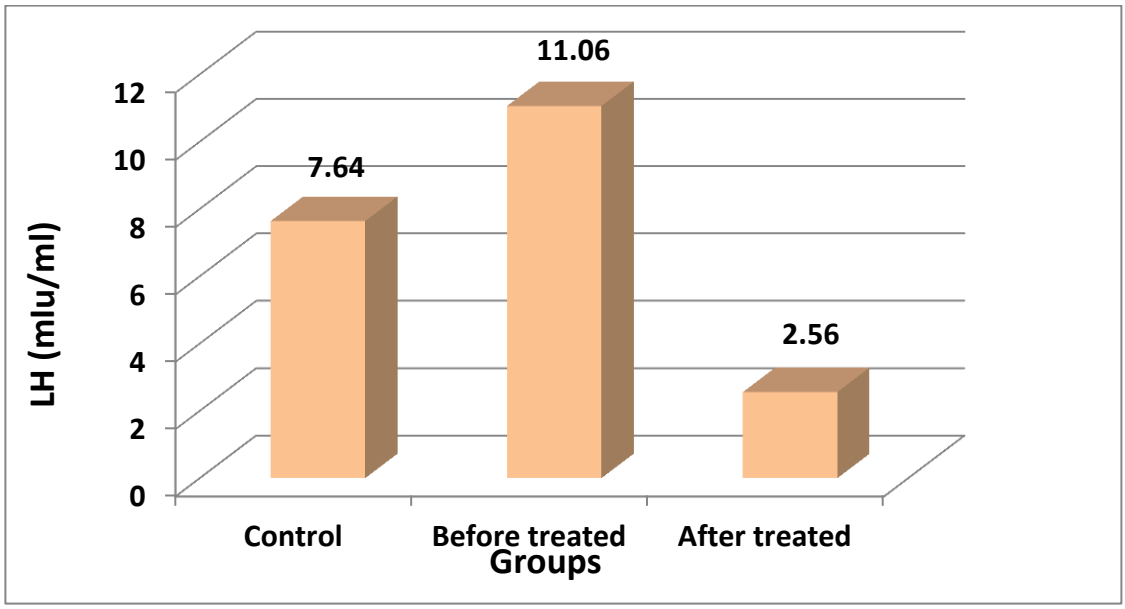

Figure (3-9): Mean values of serum LH levels in non pregnant infected women with acute toxoplasmosis before and after treatment.

From table (3-2), the results of prolactin revealed non-significance differences ($p>0.05$) between all groups of this study, figure (3-10).

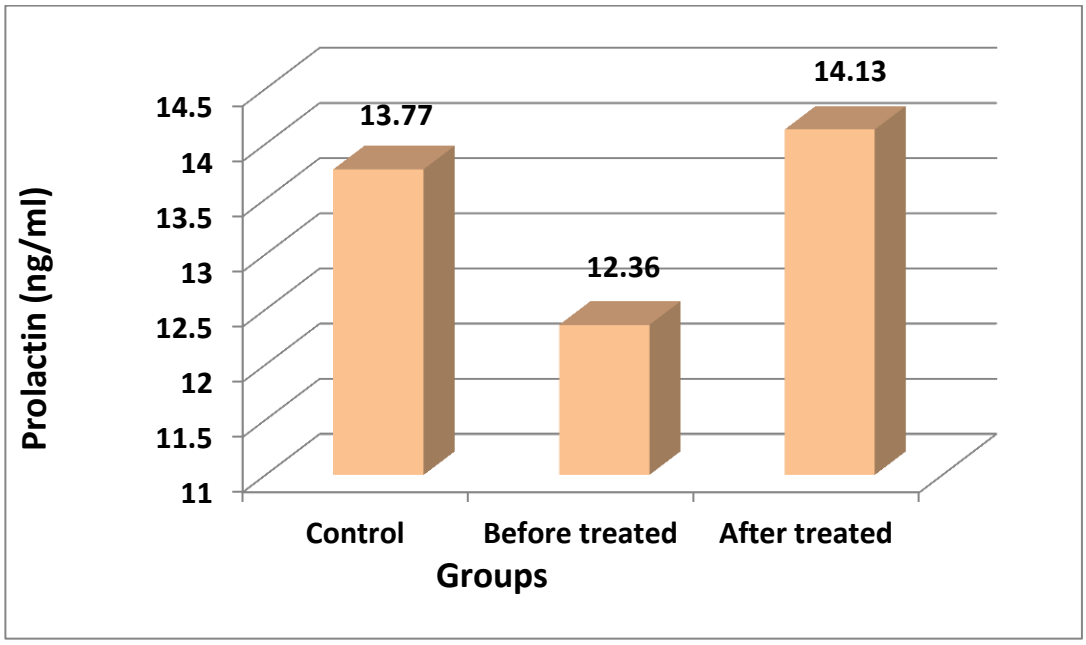

Figure (3-10): Mean values of serum prolactin levels in non pregnant infected women with acute toxoplasmosis before and after treatment.

3.3 The effect of acute Toxoplasmosis infection on the phagocytic activity in pregnant women before and after treatment:

The results of phagocytic activity of neutrophils by using nitro blue tetrazolium stain (NBT) in pregnant women infected with acute toxoplasmosis showed significant increased ($p<0.05$) in pretreated group (14.88 ± 0.46) comparing with control groups (10.45± 0.99) and post treated group (11.53± 0.41), but non - significant difference between post treatment and control groups ,table (3-3) figure (3-11).

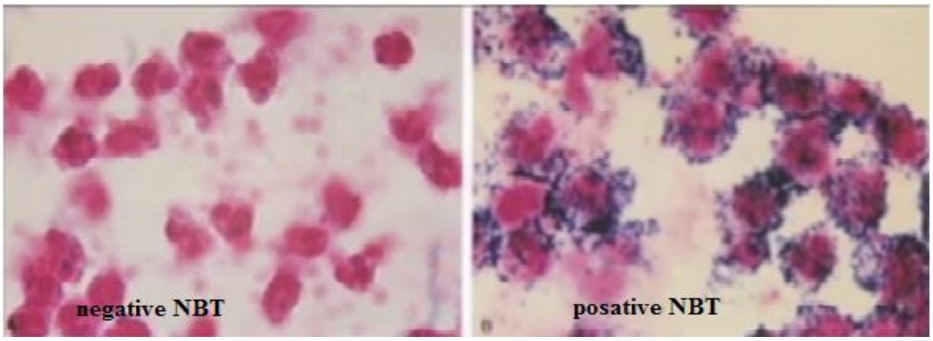

Figure (3-11): Showing Neutrophils positive by Nitro-blue tetrazolium reduction test.

Table (3-3): Phagocytic activity of neutrophil by using nitroblue tetrazolium stain in group of pregnant women infected by acute Toxoplasmosis before and after treatment:

NBT %	Groups of pregnant patients Mean ± SE				
	Control n=11	**Before treatment n=42**	**After treatment n=42**	**LSD**	**P-value**
	10.54% ±0.99 B	14.88 % ± 0.46 A	11.53% ±0.41 B	1.750 **	0.0001

Data were expressed as mean± SD; values with (A, B, C) superscript in the same group represent significance differences; ** represent highly significant difference between groups.

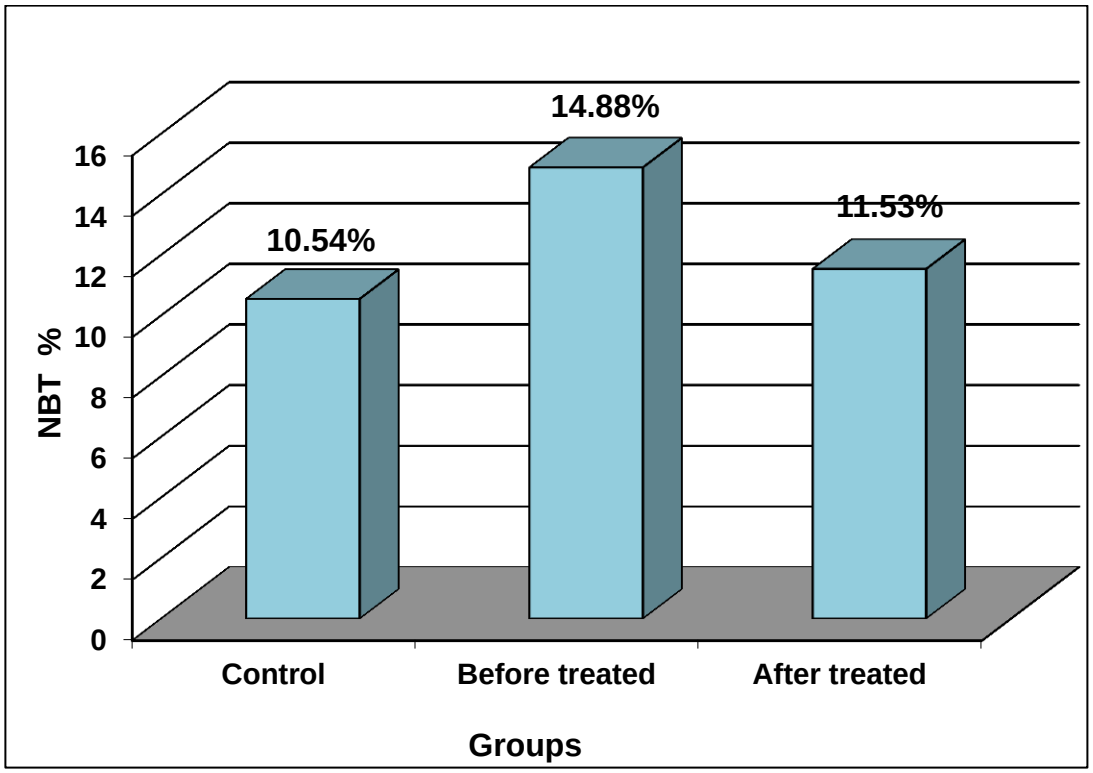

Figure (3-12): Mean changes in NBT stain in pregnant infected women comparing with after treatment group and control group.

3.4 The effect of acute Toxoplasmosis infection on the phagocytic activity of neutrophils in non pregnant women before and after treatment:

The results in table (3-4) figure (3-12) demonstrate significant increased ($p<0.05$) in the mean of active neutrophils in before treatment of non pregnant infected women with acute Toxoplasmosis (14.12 ± 0.71) compared with control groups (8.63± 0.57) and after treatment (11.82 ± 0.65), also the treated groups showed significant increased over the control group.

Table (3-4): Phagocytic activity of neutrophil by using nitroblue tetrazolium stain in group of (non pregnant) women infected by acute toxoplasmosis before and after treatment:

NBT (%)	Groups of non pregnant patients Mean ± SE				
	Control n=13	**Before treatment n=22**	**After treatment n=22**	**LSD**	**P-value**
	8.63% ± 0.57 C	14.12% ± 0.71 A	11.82% ± 0.65 B	2.115 **	0.0001

Data were expressed as mean± SD; n=number of patients; values with (A, B, C) superscript in the same group represent significance differences; ** represent highly significant difference between groups.

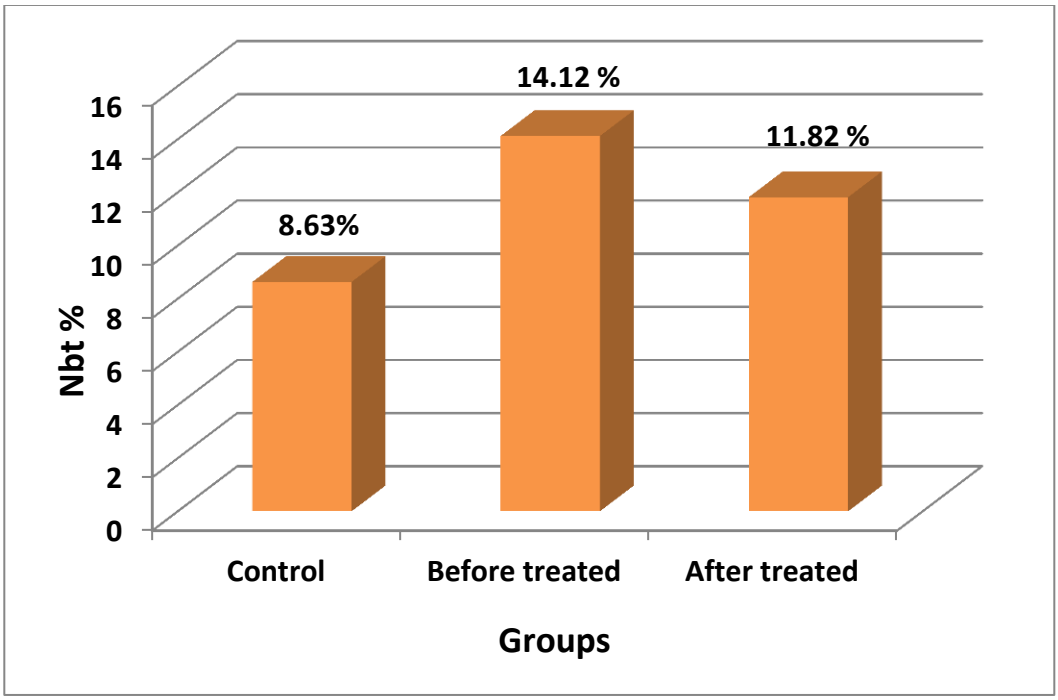

Figure (3-13): Mean changes in NBT stain in non pregnant infected women comparing with after treatment group and control group.

3.5 Hematological changes (White blood cells, Hemoglobin, Packed cell volume, Platelets) in pregnant women with acute *T.gondii* infection:

The results in table (3-5) showed the mean concentration of blood parameters (WBC, Hb, PCV, Platelets) in pregnant infected women with acute toxoplasmosis before and after treatment (spiramycin).

Table (3-5): The mean concentration of hematological parameters in (pregnant) women infected with acute toxoplasmosis before and after treated with spiramycin:

parameter	Groups of pregnant patients				
	Control N=11	before treatment n=42	after treatment n=42	LSD	P-value
WBC (cells/µL)	8654.55±449.72 A	8360.47 ± 228.19 A	8027.91 ±207.11 A	862.82 NS	0.344
Hb (g/dl)	13.18 ± 0.33 A	11.74 ± 0.20 B	12.36 ± 0.19 B	0.765 **	0.0026
PCV (%)	38.84 ± 1.18 A	35.09 ± 0.58 B	37.04 ± 0.52 AB	2.189 **	0.0040
Platelets *10^9 (cells/L)	265.00 ± 18.70 A	176.48 ± 7.88 B	171.83 ± 7.93 B	31.879 **	0.0001

Data were expressed as mean± SD; n=number of patients; values with (A, B, C) superscript in the same group represent significance differences; ** represent highly significant; NS represent Non-significant difference between groups.

The results in tables (3-5) figure (3-13) showed that mean concentration of WBC have no-significant difference in groups under study ($p>0.05$).

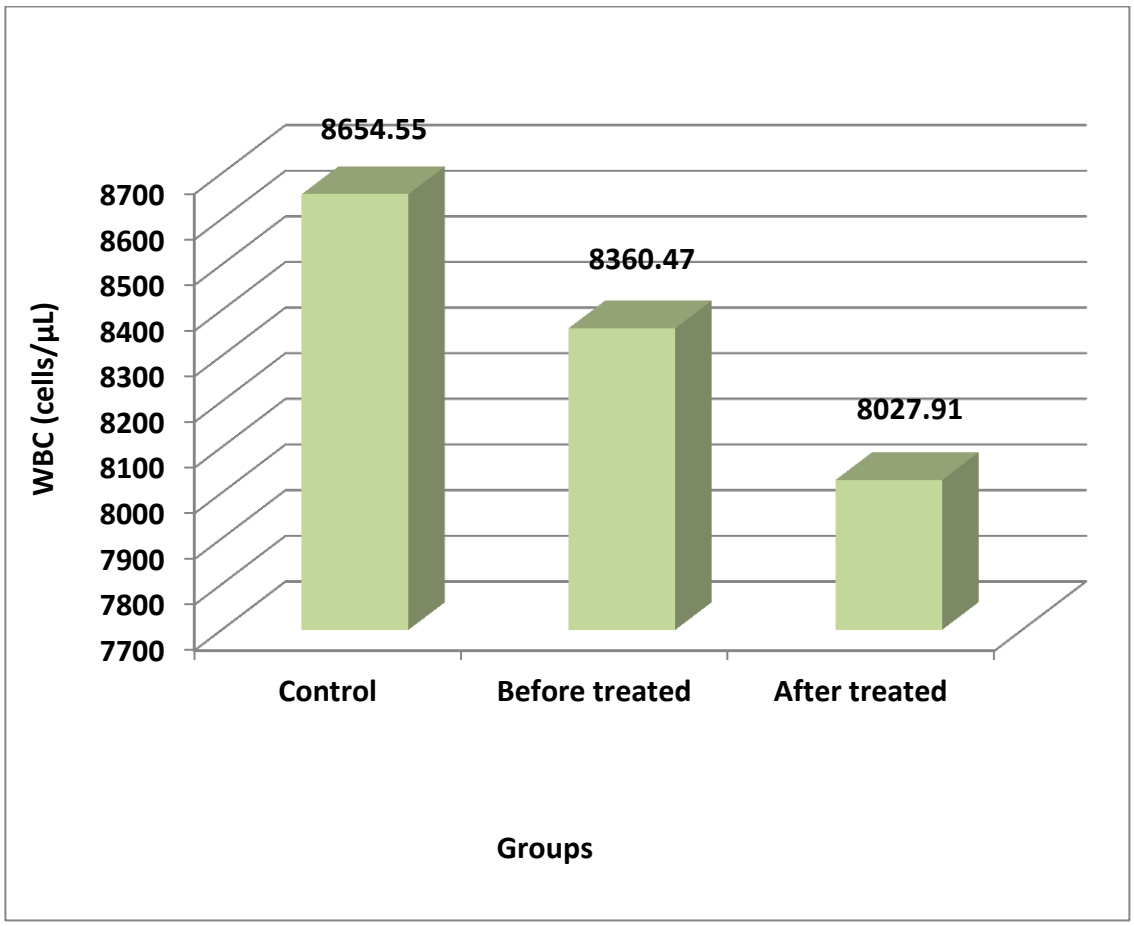

Figure (3-14): Mean values of WBC count in infected pregnant women comparing with control and after treatment groups.

While the mean concentration of hemoglobin (Hb) showed significant reduction ($p<0.05$) in the treated (12.36 ±0.19 g/dl) and pretreated groups (11.74± 0.20 g/dl) when compared with control group (13.18± 0.33 g/dl), but non-significant differences ($p>0.05$) between pretreated and treated groups, table (3-5) figure (3-14).

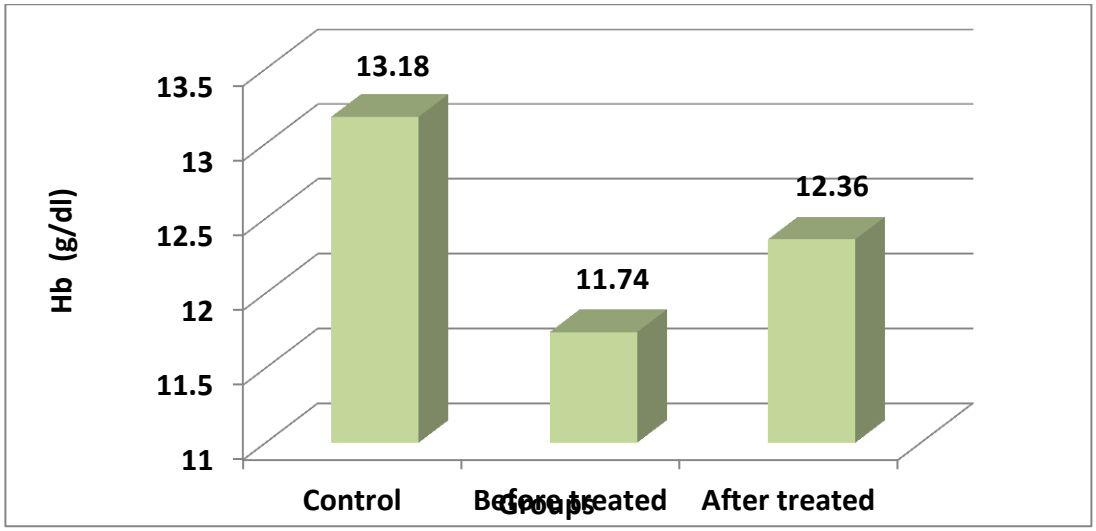

Figure (3-15): Mean values of Hb in infected pregnant women comparing with control and after treatment groups.

Also, PCV showed the same results as Hb, table (3-5) figure (3-15).

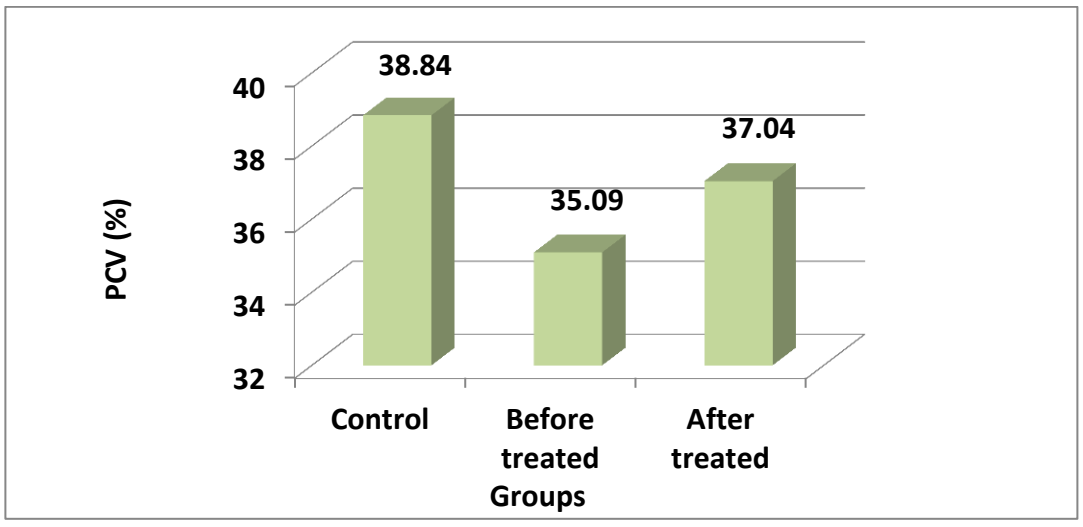

Figure (3-16): Mean values of PCV in infected pregnant women comparing with control and after treatment groups.

From table (3-5) , The result of platelets showed significant decrease ($p<0.05$) in before treated group (176.48± 7.88 cells/L) than control group (265±18.7 cells/L), also significant decreased in after treated group (171.83 ±7.93 cells/L) than in control group, But non-significant difference between before and after treated group ($p>0.05$).

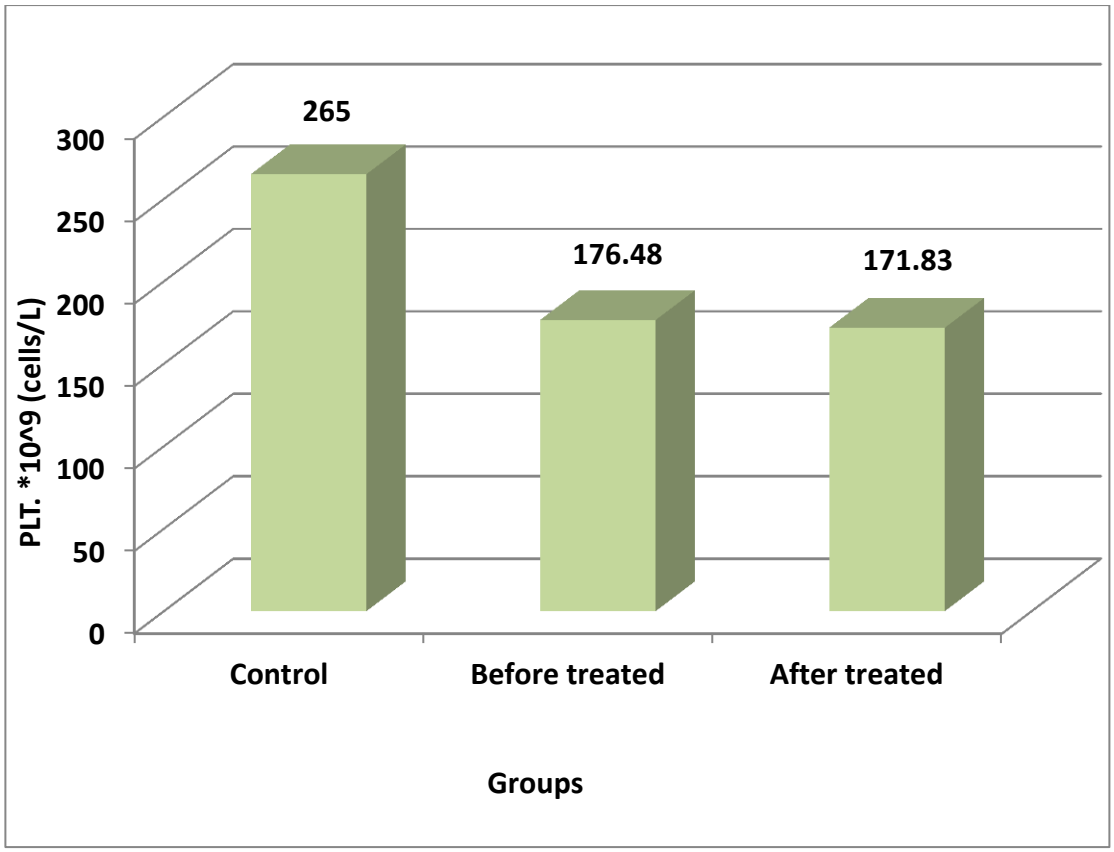

Figure (3-17): Mean values of platelets count in infected pregnant women comparing with control and after treatment groups.

3.6 Hematological changes (White blood cells, Hemoglobin, Packed cell volume, Platelets) in non- pregnant women with *T.gondii* infection:

The results in table (3-6) showed that the mean concentration of blood parameters (WBC, Hb, PCV and Platelets) in pregnant women infected with acute toxoplasmosis before and after treatment (spiramycin).

Table (3-6): The mean concentration of hematological parameters in (non pregnant) women infected with acute Toxoplasmosis before and after spiramycin treatment:

parametr	Groups of non pregnant women				
	Control n=13	before treatment n=22	after treatment n=22	LSD	P-value
WBC (cells/µL)	7509.09 ± 263.69 B	8691.67 ± 177.33 A	8331.82 ± 178.66 A	592.64 **	0.0017
Hb (g/dl)	12.97 ± 0.35 A	12.11 ± 0.29 A	12.35 ± 0.22 A	0.869 NS	0.181
PCV (%)	40.57 ± 0.96 A	35.94 ± 0.76 B	37.72 ± 0.65 B	2.346 **	0.0019
Platelets *10^9 (cells/L)	308.18 ± 24.36 A	283.79 ± 19.85 A	186.22 ± 10.74 B	54.491 **	0.0001

Data were expressed as mean± SD; n=number of patients; values with (A, B, C) superscript in the same group represent significance differences; ** represent highly significant; NS represent Non-significant difference between groups.

Data presented in table (3-6) and figure (3-17) showed that there is significance increased of WBC levels (P<0.05) before treated non –pregnant women infected with acute *T.gondii* (8691.67 ± 177.3 cells/ µL) comparing with control groups (7509.09± 263.69 cells/ µL), also significant increased after treated groups (8331.67 ±178.66 cells/ µL) than control group, But non-significant differences between treated and before treated groups was found.

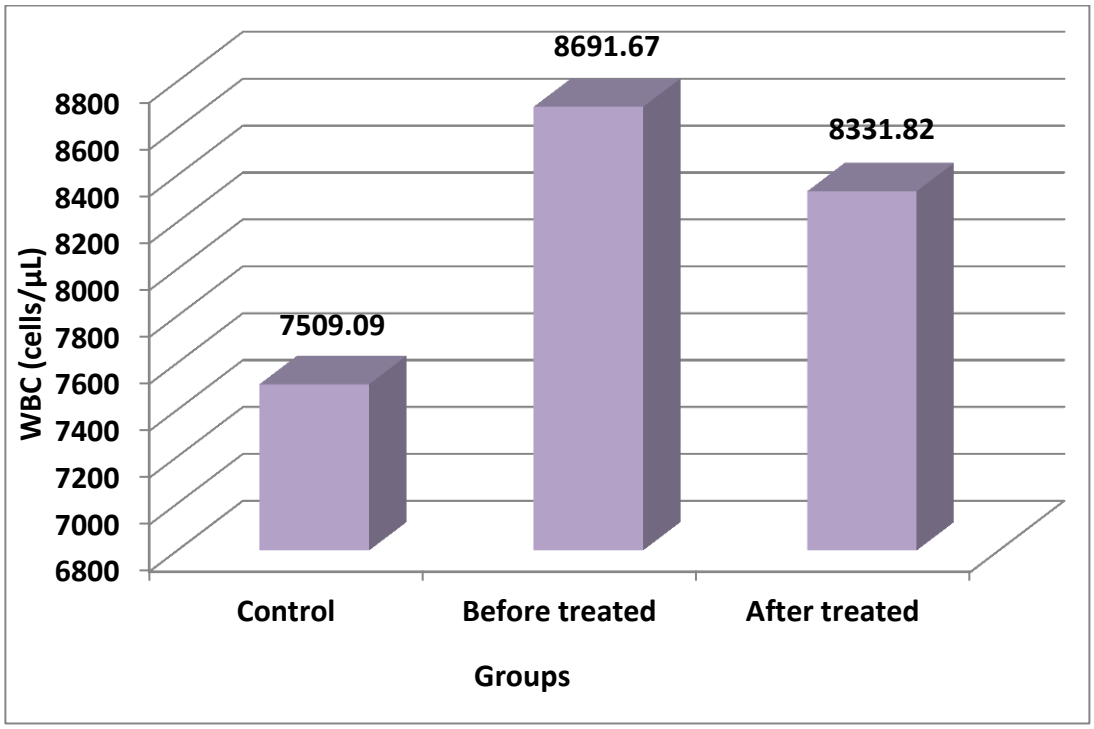

Figure (3-18): Mean values of WBC count in infected non pregnant women comparing with control and after treatment groups.

The result of Hb as mentioned in table (3-6) figure (3-18) showed non significant difference (p>0.05) between all study groups.

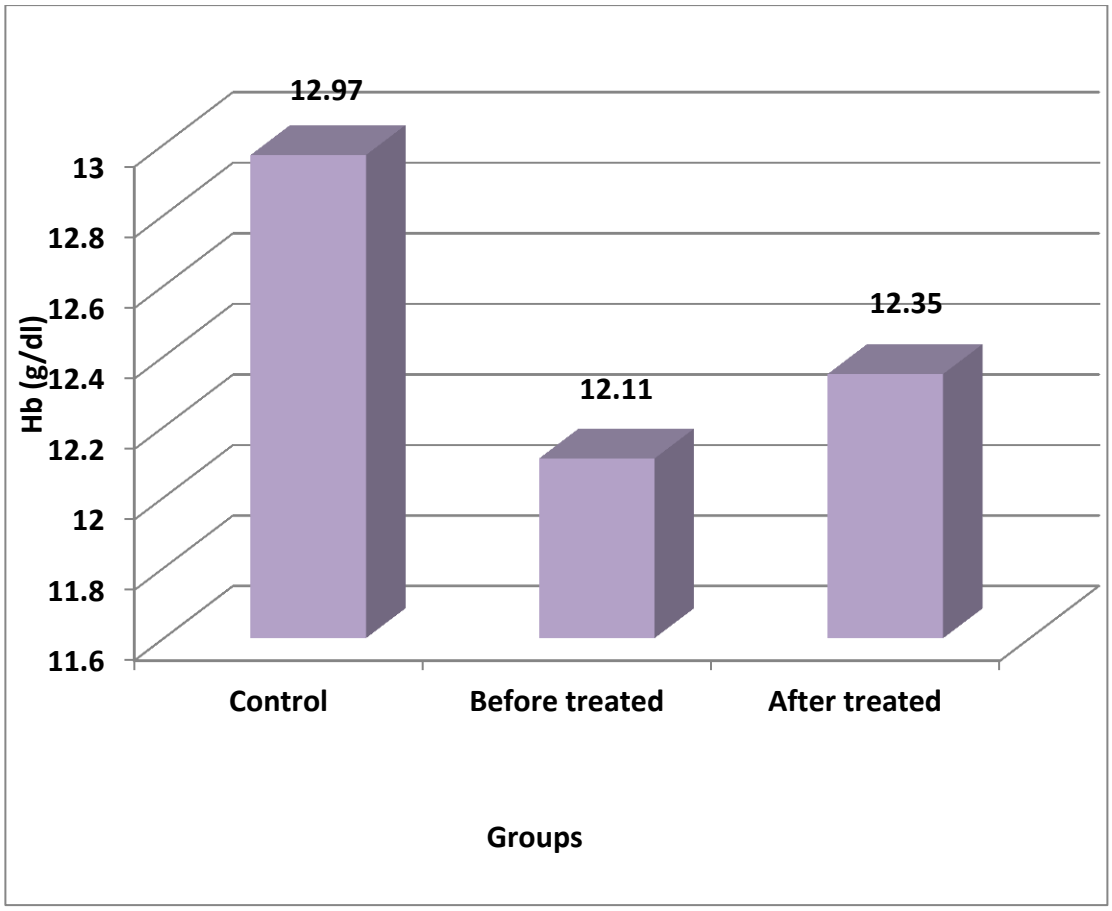

Figure (3-19): Mean values of Hb in infected non pregnant women comparing with control and after treatment groups.

The result of PCV showed significant reduction ($p<0.05$) before treated group (35.94± 0.76) than control group (40.57± 0.96), and also significant decreased ($p<0.05$) after treated group (37.72 ± 0.65) when compared with control. Whereas non – significant differences ($p>0.05$) between before and after treated groups .Table (3-6) ,figure (3-19).

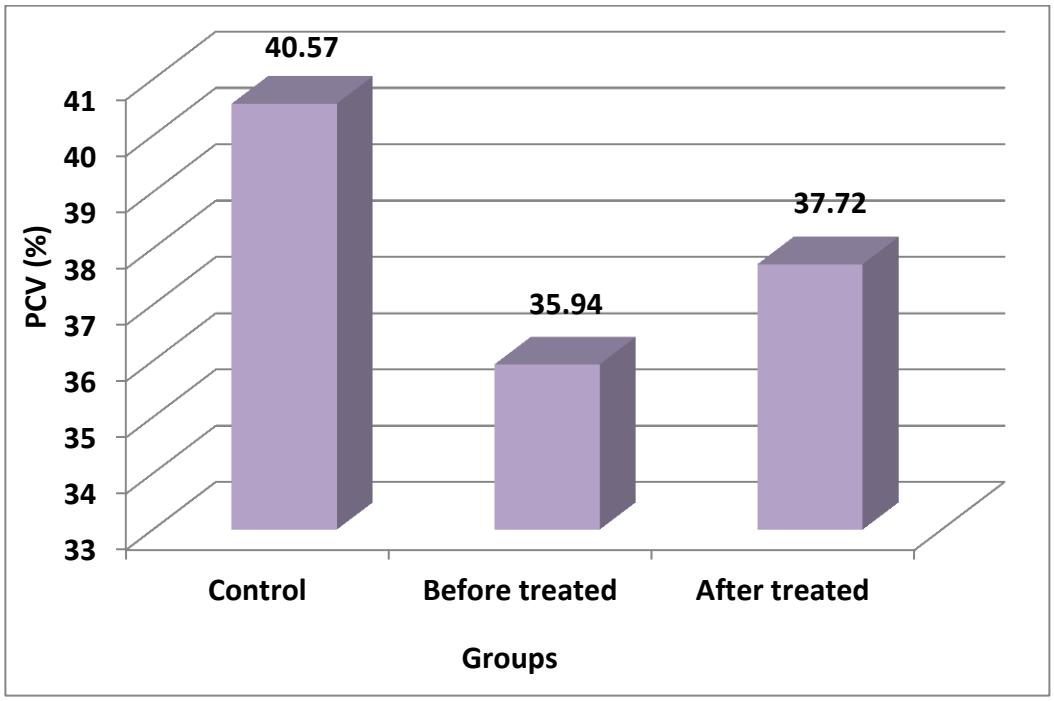

Figure (3-20): Mean values of PCV in infected non pregnant women comparing with control and after treatment groups.

Platelets results showed non – significant decreased ($p>0.05$) in before treated group (283.79 ±19.85 cells/L) compared with control group (308.18 ± 24.36 cells/L) but significant decreased ($p<0.05$) in after treated group (186.22 ± 10.74 cells/L) comparing with control and before treated groups.

Table (3-6); figure (3-20).

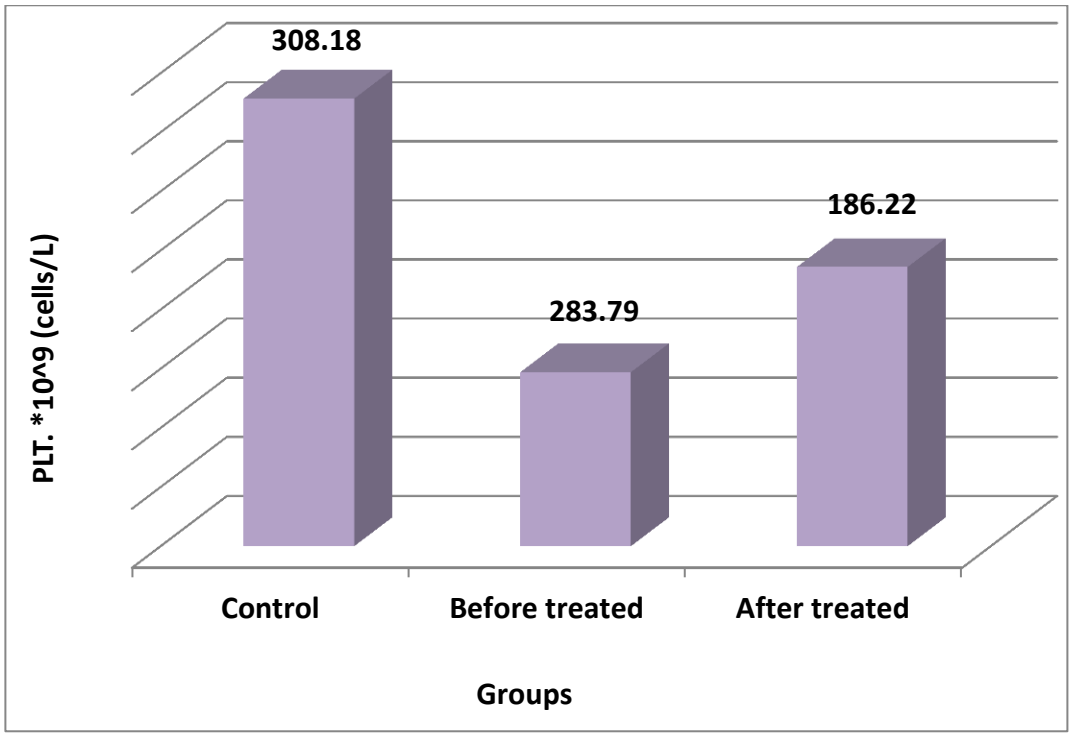

Figure (3-21): Mean values of platelets count in infected non pregnant women comparing with control and after treatment groups.

Chapter Four

Discussion

4.1 The effects of acute *Toxoplasma gondii* infection on the hormonal levels of both pregnant and non pregnant women before and after spiramycin treatment:

There is evidence that *T.gondii* infection is affected by sex hormones levels, as a result, hormonal changes can affect the immune system in general. Thus, it increases the possibility of disease, and particular immunity to selective parasitic disease such as *Toxoplasma gondii*. The importance of this parasite is because of the ability of *T.gondii* to cause congenital disease if toxoplasmosis infection happened throughout pregnancy [(81)].

Toxoplasmosis is one among the classical conditions that possessed profound adverse effects on function of the reproductive system in human [(135,136)]. In females there is a fluctuation in sex hormones (estrogen and progesterone) due to variation in concentrations of pituitary follicle stimulating hormone (FSH) and luteinizing hormone (LH) during menstrual cycle[(137)]. These variations include: increase in 17 beta-estradiol and decrease in progesterone plasma level during follicular phase and in the luteal phase high plasma 17 beta-estradiol and progesterone levels, respectively, so these variations in hormonal levels may affect the immune system that leads to increase the susceptibility to Toxoplasmosis infection [(138)].

4.1.1 Estrogen:

The results of Estrogen shows a significant increase ($p<0.05$) in pregnant women infected with acute *T.gondii* before treatment when compared with control and post treated group, table (3-1) and figure (3-1).

This result agrees with other studies who found high level of estrogen in infected women with *T.gondii* compared with non infected women [85,139]. But another study by Omima *et al.* showed a significant decrease in serum estrogen hormone in aborted women infected with *T.gondii* [140]. This result may be was seen after chronic infection; while the present study includes patients in the acute phase of *Toxoplasma* infection.

The result of the current study in non pregnant infected women showed significant increase in level of estrogen in a pretreated group compared with after treated and control group, table (3-2) figure (3-6). These results, in compared with the results of study by Omima *et al.* found that there is reverse difference, because Omima *et al.* reported that there is significant decreased in serum estrogen hormone in aborted women infected with *T.gondii* compared to control group [140].

The findings of the present study concerning non pregnant women may be attributed to that women are subjected to regular cyclic changes in hormones level (estrogen & Progesterone). These changes occurs mostly as a physiological fluctuation during menstrual cycle, these hormones has a large effects on cells involved in immune system, and as a sequence of this effect on immune system will increase susceptibility to *Toxoplasma* infection [81].

4.1.2 Progesterone:

The result of progesterone revealed non- significant difference between the study groups in pregnant infected women, table (3-1) and figure (3-2). These results agreed with Al-Warid HS *et al.* who also found that there were no significant difference in progesterone levels between infected pregnant women and non infected [85].

In addition, the results of the current study agree with Kadhim.R *et al.* who reported that there is no significant difference in progesterone level in first trimester. But disagree with the finding that concerning the women in second trimester in which there is significant decrease in progesterone level in seroposative IgG women. Also with women in the third trimester of pregnancy in which there is significant increase in level of progesterone in seroposative IgG[141]. This may be explaining the finding of present study which includes patients with seroposative IgM.

The progesterone level in the groups of present study with non pregnant women showed that there were no significant differences in levels of progesterone between infected and non infected women with acute *Toxoplasma* infection. Although low progesterone levels were noticed in infected women compared with higher level in non infected women (control group). (2.78 ± 1.01pg/ml) (3.65 ± 1.80 pg/ml) respectively. As showed in table (3-2) and figure (3-7).

Progesterone can have both stimulatory and suppressive effect on the immune system, but it's typically regarded as immunosuppressive [82]. In the present study it seems that the high progesterone level in non infected have

immuno stimulant effect characterized by higher cellular immunity [81] which can overcome the *T.gondii* proliferation.

4.1.3 Follicle stimulating hormone (FSH):

The results of Follicle stimulating hormone (FSH) showed significant increase in pregnant and non pregnant infected women before treatment compared with after treatment and control group.

The similar finding of the current study also recorded by Al-Warid HS *et al.* who reported high level of FSH hormone in pregnant women infected with *T.gondii* [142], due to the correlation of some sex hormones with the immune response; i.e. high concentration of sex hormones correlate with low immune response, which may lead to increase susceptibility to parasitic infection [82].

But the result of the current study do not agree with the finding of Oktenli C *et al.* in Turkey who reported that temporary hypogonadotrophic gonadal insufficiency might occur due to acute *Toxoplasma* infection despite the course of the disease and found that level of IL-1B was higher in infected patient with Toxoplasmosis who have low FSH level[97]. This disagreement may be related to the differences in the ethnic group, environmental condition and nutritional factors which may cause the difference in susceptibility to infection and in the concentration of sex hormones.

Moreover, experimental studies on mice demonstrated that mice undergo reproductive failure due to dysfunction of hypothalamus resulting from chronic *Toxoplasma* infection [143], where as the results of the present study are in the acute phase of the *Toxoplasma* infection.

4.1.4 Luteinizing hormones (LH):

The results of the present study show the opposite results in outcomes among pregnant and non-pregnant, which found that there is no significant difference in LH hormone level between the groups under the study in pregnant infected women with toxoplasmosis (table 3-1) and (figure 3-4).

While there is a significant increased ($p<0.05$) in the LH level in non-pregnant infected women before treatment compared with after treatment and control groups. But significant decreased in after treatment group comparing with before treatment and control groups, (table 3-2) (figure 3-9).

These results were similar to the results of Al Warid SH *et al.* who found that *Toxoplasma* infection may not lead to an increase in LH in pregnant infected women [142]. That because during pregnancy the FSH and LH levels suppressed by the high concentration of estrogen and progesterone and become undetectable during pregnancy [143, 121].

As well as , the finding of the current study concerning the pregnant infected women correlate with the finding of Rui Y *et al.* who showed there were no obvious changes in LH hormone in mice infected with *T.gondii*[144].

On the other hand, the present data concerning the non pregnant infected women with *Toxoplasma* were similar to Dvorakova-Hortova K *et al.* who also observed a significant difference of LH level in mice urine before and after infection [145]. *T.gondii* may activate hypothalamus-pitutary-adrenal (HPA) stress axis and consequently modify the hypothalamus-pitutary-gonadal (HPG) axis, result in alteration of gonadotropins (LH, FSH) release, so this mechanisms may explained the difference in LH level during the infection [145].

4.1.5 Prolactin hormone:

The results of prolactin levels in the serum of both pregnant and non-pregnant infected women not differ significantly ($p>0.05$) between the study groups. This may be attributed to that level of prolactin increased as the pregnancy proceeds to the third trimester and reach the peak at parturition. As well as Prolactin level drops during active labor, then rise again after delivery [(146)], however the patients in the present study were pregnant and non pregnant women in the first trimester of pregnancy.

The data obtained from present study were disagree with previous study by Kadhim.R *et al.* who reported that chronic *toxoplasma* infection in pregnant women associated with variation in the prolactin levels and the highest value recorded in the third trimester [(141)].Whereas the patients of current study were pregnant women infected with acute toxoplasmosis and all of them in the first trimester of pregnancy.Also another study by Dzitko K *et al.* demonstrate that the level of prolactin significant decrease in all positive IgG antitoxoplasma Ab pregnant women[(103)], but the present study includes only patients with antitoxoplasma Ab IgM.

The relatively higher levels of prolactin hormones in seronegative groups (control group) than seropositive groups (acute infection) may be a sign of the prolactin protective action in a host against *T.gondii* infection. It has been reported that prolactin hormone increases the production of immune globulins, cytokines and auto antibodies [(147)]. Prolactin could be one of the humoral factors that probably limit the invasion of *T. gondii*. The results propose that physiological elevation in prolactin level throughout gestation may reduce the risk of *Toxoplasma* infection [(104)].

4.2 Phagocytic activity of neutrophils during *T.gondii* infection:

The results of phagocytic activity of neutrophils by using nitroblue tetrazolium (NBT) stain in both pregnant and non-pregnant women revealed significant increase of NBT positive neutrophils count in infected women with *T.gondii* before treatment than after treatment and control group, table (3-4) figure (3-12). The efficiency of neutrophils as phagocytes is measured by nitro-blue tetrazolium (NBT) test [(148)]. The nitro-blue tetrazolium dye is converted by the reduction occurring during phagocytosis to an insoluble blue-black formazane deposit [(127, 148)].

In normal individuals (control group) the percentages of NBT positive neutrophils (with formazane deposits) was reported to vary between 3% to11 % [(127, 148)]. An increase in the positive NBT% was reported in the majority of bacterial and parasitic infections [(127, 148, 107)] and may reach up to 75% in acute phase of infection and then decline gradually until return to normal values within 4-6 weeks. Neutrophils have an essential role in the non-specific (innate) immunity of the body [(149)], its increase means that this kind of immunity is efficient.

The results of present study are basically in agreement with Ismail M *et al.* results, which showed that significant increase in phagocytic activity of polymorph neaucler neutrophils (PMNs) for reduction of NBT stain[(150)].

On the other hand Aghwan S S *et al.* showed that there is a significant increase in the NBT positive neutrophil count in rats infected with *Toxoplasma gondii* comparing with non infected group, but observed significant decrease in the rate of PMNs viability that isolated from peripheral blood of infected rat compared with control group[(151)]. The reduction in cell

viability attributed to parasitic toxins which inhibit enzyme activity of ATPase[150].

The present study showed that NBT test could be efficient and helps in the determination of the phase of infection whether acute or chronic because its level reflected the innate immunity which considered important in the diagnosis of the disease. Also NBT test could be used as follow up test to evaluate the degree of treatment efficiency and patient's compliance [107].

4.3 The effects of acute *Toxoplasma gondii* infection on the hematological parameters in the sera of both pregnant and non pregnant women before and after treatment:

With regards to the effect of toxoplasmosis on some blood components in women comparing before and after treatment, the results show alteration in levels of hematological parameters.

4.3.1 White blood cells:

The statistical analysis of the present study demonstrates a non significant differences ($p>0.05$) in leukocyte count among all study groups (control, treated, post treated) in pregnant infected women with *T.gondii*, table (3-5) figure (3-17). While, in non pregnant infected women showed significant increased ($p<0.05$) in pretreated and after treated groups in compared with control group, table (3-6) and figure (3-17).

White blood cells were measured in order to evaluate the inflammatory response stimulation in *T.gondii* infected patients, and to correlate this inflammatory response with duration of infection.

The findings of current study correlate with Anwar AK *et al*. findings who pointed that WBC count show higher value with significant difference in infected women compared with non- infected women with *T.gondii*[152].

The elevated level of leukocyte count may be attributed to the ability of WBC (especially eosinophils) to destroy the parasite by attachment and granule secretion [153] as immune response of body to destroy the parasite, so the cellular immunity will activated plus the humoral immunity [154].

The present study revealed that there was no significant difference ($p>0.05$) in WBC count between infected and non – infected pregnant women with toxoplasmosis. Although lower level of leukocyte are observed in infected women. However; other reports demonstrated a significant decrease in total leukocyte count in human serum positive for anti *Toxoplasma* antibodies[155]. But another study by Hamad HK *et al.* found that there is significant increase in WBC level in blood of pregnant women infected with acute Toxoplasmosis [156].

4.3.2 Hemoglobin:

This study showed that the results of Hemoglobin (Hb) in pregnant infected women significantly decreased ($p<0.05$) in before and after treatment patients when compare with control group. While the data of Hb obtained from non-pregnant infected women appeared non- significant difference between the study groups ($P>0.05$), table (3-6) figure (3 -18).

The findings of the present study concerning the pregnant infected women agreed with other studies who showed significant decreased in Hb

level during acute toxoplasmosis in the blood of pregnant infected women compared with control group [156,152,157]. The reduction in Hb level may be correlate with many factors: like parasitic effect on the body cells and multiplication of the parasite [120]. Also may be due to degradation of red blood cells which may cause anemia. Moreover during pregnancy the plasma undergoes relatively slight increase compared with RBC which may result in hemodilution which called physiological anemia of pregnancy [121].As well as, anemia accompanied with enlargement of lymph node usually occurred during toxoplasmosis infection due to hepatomegaly and hepatitis [158].

Although, the result of Hb level in non- pregnant women not differ significantly ($p>0.05$) between infected and non infected group, but lower level of Hb were noticed in women infected with *T.gondii* (12.11± 0.29 g/l) and (12.97± 0.35 g/dl) respectively.

This result agreed with study of Shelly S *et al.* and Al- Nasiri AR *et al.* who reported a significant decrease in Hb level in non pregnant women infected with acute *T.gondii* comparing with control group [155, 159]. Reduction in Hb concentration may be attributed to multiplication of parasite inside the host body [120].

4.3.3 Packed cell volume (PCV):

The results of pact cell volume in both pregnant and non pregnant infected women with *T. gondii* showed significant reduction of PCV ($p<0.05$) in infected group comparing with control group (35.09± 0.58 , 38.84± 1.18) for pregnant women , and (35.94 ± 0.76 , 40.57 ± 0.96) respectively for non pregnant women ,Table (3-5) and figure (3-19).

The results of present study were similar to the results of other studies who showed that PCV level were below the reference range in patients infected with *T.gondii* infection and suggested possible development of anemia[152,157,159], which may occur due to degradation of RBC in the infected tissue with *T.gondii*[160] . Also during pregnancy may develop anemia due to fetal placental demand for iron with the maternal plasma volume expansion which result in reduction of PCV [161].

4.3.4 Platelets:

The results of Platelets from table (3-5) and figure (3-16) showed a significant decrease ($p<0.05$) in pregnant infected women with acute toxoplasmosis before and after treatment compared with control group. (176.48 ±7.88 cells/L),(171.83 ± 7.93cells/L) and (265 ± 18.7 cells/L) respectively.

Whereas the results of platelets count in non-pregnant infected women with *T.gondii* was decreased before treatment (283.79 ± 19.85 cells/L) and significant decreased after treatment group when compared with control group (186.22 ± 10.74 cells/L , 308.18 ± 24.36 cells/L) respectively .

The current results agreed with a previous study who reported that thrombocytopenia during acute toxoplasmosis in the blood of pregnant women[156]. This reduction of platelet count may attributed to the effects of parasite on the host body cells[120]. As well as, pregnant women undergo physiological changes during pregnancy result in elevated platelets activation and accelerated clearance this is term as (gestational thrombocytopenia) [50].

Also aonther study by Young EC *et al.* observed thrombocytopenia in *T.gondii* experimentally infected rats [163]. On the other hand researchers found in many cases of congenital toxoplasmosis that thrombocytopenia was combined with these cases [164]. Additionally, any protozoal or bacterial infection that related to thrombocytopenia may result from different mechanisms like: increase the clearance of platelets that damaged by (exotoxins and endotoxins), or platelet undergo immune-mediated destruction, and direct platelet toxicity caused by the parasite [15].

Platelet play important role in the defense mechanism of the host against *T.gondii* infection by aggregation, interaction then release of their products. Platelets need close contact between target cells and effector to induced killing of this parasite through adherence of platelets pseudopodial projections to the surface of the parasite, then swelling of the outer membrane of the parasite which results in intracellular cytolysis of *T. gondii* [113].

Conclusions and Recommendations

4.4 Conclusions:

Based on the obtained results, it is possible to reach the following conclusions:

1- Estrogen level in pregnant patients recorded a significant elevation in both pretreated and post treated group in comparison with control group, whereas FSH hormone level recorded higher mean concentration in pre treated group.

2- Estrogen level in non pregnant patient's recorded high significant mean concentration in pretreated group in comparison to post treated and control group. While FSH and LH revealed significant increase in pretreated group, but significant decrease in post treated group.

3- Neutrophils activity by using (NBT) stain in pregnant women revealed a significant difference in the groups of the study. The higher percentage of neutrophils activity was recorded in pretreated group compared to the other groups. While, in non-pregnant women, the phagocytic activity of neutrophils recorded significant increase in pretreatment and post treatment when compared with control group.

4- The hematological parameter (Hb, PCV, and Platelet) in pregnant infected women recorded a significant difference between study groups. Hb scored significant reduction in both pretreated and post treated compared to control, also platelets recorded the same results as Hb. While PCV scored the lowest value in pretreated group.

5- In non-pregnant patients, the hematological parameters (WBC, PCV, and Platelets) revealed significant differences between the study groups. WBC recorded significant elevation in both pretreated and prost treated group when compared with control, whereas PCV recorded significance

reduction in pretreatment and post treatment than the control group. And platelets scored the lowest value in post treated group.

6- The *Cobas e411* analyzer represented rapid and accurate diagnosis which is essential to ensure effective treatment and prevention of development of the disease.

4.5 Recommendations:

The presented conclusions may permit to suggest the following recommendations:

1- More investigations in detection the number and location of genes by purification of protein (gene) which is useful for development of gene vaccine.

2- Further studies focusing on mother immunity to *Toxoplasma* during pregnancy because it plays a vital role as a defense mechanism against the parasitic infection

3- It would be useful to study with larger sample size and larger follow up to specific evaluate the hormonal variation, as well as to clarify the etiology of this variation for better management.

4- Work on the development of health awareness programs to reduce risk of the disease on the community especially pregnant women, and work on the making the test for Toxoplasma antibodies as a routine test for all pregnant women.

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Appendices

Questionnaire sheet used for each female included in this study:

Name: ..... Age:.....

Address: ..... date:.....

Pregnant (month): not pregnant:

Last menstrual period:

No. of pregnancy: No. of children:

No. of abortion: No. Of still birth:

No. of congenital anomalies:

History of chronic disease: Yes ( ) No ( )

Is there is a previous Toxo test? Yes ( ) No ( )

if yes +ve ( ) -ve ( )

Any previous treatment for +ve cases? Yes ( ) No ( )

Period of treatment & dose:

الخلاصة

المقوسة الكوندية هو المسبب لداء المقوسات الذي يصيب نسبة كبيرة من سكان العالم، وخصوصا النساء الحوامل فقد يسبب الاجهاض واضرار جسيمة للجهاز العصبي في الجنين. أجريت هذه الدراسة لمعرفة تأثيرالاصابة الحادة بطفيلي المقوسة الكوندية*T.gondii* على مستويات الهرمونات (الاستروجين والبروجسترون، FSH، LH والبرولاكتين) وكذلك تاثير الطفيلي على المستويات الدموية والتي تشمل (هيموغلوبين ،صفائح الدم،كريات الدم البيضاء، هيماتوكرت)، وقياس نسبة نشاط الخلايا البلعمية العدلة باستخدام صبغة (NBT) في النساء الحوامل (في الاشهر الثلاثة الاولى من الحمل) و غير الحوامل المصابات بداء المقوسات الحاد.شملت هذه الدراسة 64 امرأة مصابة و 24 امرأة سليمة كمجموعة سيطرة، المراجعات الى مستشفى الكوت للولادةفي محافظة واسط للفترة من ايلول 2014 إلى ايار2015 واعطاء النساء المصابات علاج السبايرامايسين لمدة شهرين. أظهرت النتائج أن هنالك علاقة بين الاصابة بداء المقوسات الحادمع زيادة معنوية في مستويات كل من هورمون الاستروجين (34.42 ± 518.67بيكوغرام / مل) و هورمون FSH(0.735 ± 0.24 ميكرو وحدة / مل) للنساء الحوامل و (132.37 ± 9.94 بيكوغرام / مل) و ± 9.08 (3.19ميكرو وحدة / مل) لغير الحوامل على التوالي .كما ان الاصابة الحادة بطفيلي المقوسة الكوندية تحدث انخفاض معنوي لهورمون LH في النساء غير الحوامل فقط (11.06 ±3.95 ميكرو وحدة / مل)، في حين لم تسجل أية تغييرات معنوية لهورمون البروجستيرون والبرولاكتين في كلا المجموعتين من النساء.كذلك اظهرت الدراسة ان الخمج بطفيلي المقوسة الكوندية يحدث زيادة معنوية في نشاط الخلايا العدلة في النساء المصابات بالمقارنة مع مرحلة ما بعد العلاج ومجموعة السيطرة في كلا من النساء (الحوامل وغير الحوامل).كما بينت نتائج الدراسة ان هنالك انخفاض معنوي في مستوى الهيموغلوبين وانخفاض في مستوى PCVوكذلك انخفاض عددالصفائح الدموية لدى النساءالمصابات(الحوامل)فقط عند مقارنتها بمجموعة السيطرة.بينما في النساء المصابات من(غير الحوامل) أظهرت زيادة معنوية في مستويات كريات الدم البيض والصفائح الدموية فقط بينما انخفاض معنوي في مستوى PCV.

يمكننا أن نستنتج أن الإصابةالحادة بطفيلي المقوسة الكوندية في النساء الحوامل وغير الحوامل ترتبط بتغييرات في مستويات بعض الهرمونات وهذه التغييرات قد تؤثر على جهاز المناعة وبالتالي زيادة القابلية للإصابة بالخمج .

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

الجامعة المستنصرية

كلية الصيدلة

التغيرات الهرمونية والدموية في النساء المصابات بداء المقوسات خلال سن الانجاب قبل وبعد اعطاء السبايراماسين

رسالة مقدمة الى فرع العلوم المختبرية السريرية والى لجنة الدراسات العليا في كلية الصيدلة- الجامعة المستنصرية كجزء من متطلبات الحصول على درجة الماجستير في الصيدلة (العلوم المختبرية السريرية)

من قبل

رغـدة هشــام خليــل الجـوراني

بكلوريوس صيدلة 2010

باشراف

أ.م.د جابر حميد حسين

أ.م.د محمد الاعرجي

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2015 م