Journal Homepage: -www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI:10.21474/IJAR01/1762
DOI URL: http://dx.doi.org/10.21474/IJAR01/1762
ISSN NO. 2320-5407

RESEARCH ARTICLE

IMMUNOHISTOCHEMICAL AND HISTOPATHOLOGICAL CHANGES OF SQUALENE AS AN ADJUVANT.

Fatma A. Eid, Maha G. Soliman and Alya M. Aly.
Department of Zoology, Faculty of Science, Al- AzharUniversity, Cairo, Egypt.

Manuscript Info

Manuscript History

Received: 12 August 2016
Final Accepted: 19 September 2016
Published: October 2016

Key words:-
Squalene
Immunohistochemistry
Proliferating cell nuclear antigen(PCNA)
Spleen

Abstract

Vaccination is a public health measure intended to reduce the incidence of infectious diseases. Just few years ago, the term "adjuvant" was officially linked to vaccine and plays a key role in boosting immunogenicity. Squalene as adjuvant of vaccine enhances antigen-specific immune responses and expand coverage through dose sparing reducing amount of vaccine usage. The objective of this study is to investigate the possible immunohistochemical activity of cell proliferation and histopathological effects of squalene as an adjuvant of the spleen. Albino rats were injected with two doses of squalene (AS03) at interval three weeks between them. Results obtained in the present study showed that squalene as adjuvant contributed to magnification of immune response, exemplified by increasing proliferating cell nuclear antigen in immune cells. Squaleneoverstimulates the splenic tissue where they direct the type, magnitude and quality of the adaptive immune response, rather than some histopathological observations. Long period group has adverse events that showed slowly recovery after the squalene treatment.

Introduction:-

The theory of stimulating the body's immune response is the basis underlying vaccination. Vaccination not only provides protection against the pathogens itself but also prevents the prolonged immunosuppression that occurs as a consequence of this disease (Mina *et al.*, 2015). Vaccines act by initiating the innate immune response and activating antigen presenting cells, thereby inducing a protective adaptive immune response to a pathogen antigen. The vaccines that deliver intact microorganisms may be enhanced by an additional adjuvant (Helen *et al.*, 2016). Adjuvants are substances added to vaccines to enhance the immunogenicity of highly purified antigens, Oil-in-water adjuvants (squalene) can boost humoral responses to seasonal vaccines, but relatively little is known about their mechanism of action (Karen*et al.*, 2016). Pandemic-influenza vaccines containing split-inactivated-virus antigen have been formulated with the immunostimulatory adjuvant system AS03 to enhance the antigen immunogenicity and reduce antigen content per dose. The administration of repeated doses of the AS03 vaccine was primarily associated with transient mild inflammation at the injection site and draining lymph nodes. The biodistribution kinetics of AS03 constituents were consistent with AS03 inducing this pattern of inflammation (Segal*et al.*, 2015). The squaleneadjuvantation increased pain at the site of injection and increased unsolicited adverse events, erythema, induration and swelling at the injection site, which elicited significantly higher immune response (Della*et al.*, 2014), followed by tissue necrosis, intense inflammation and granulomatous lesion (Viera, 2001). Adjuvants can break tolerance meaning and they can disable the immune system to the degree that it loses its ability to distinguish what is

Corresponding Author:-Alya M. Aly.
Address:-Department of zoology, faculty of science, al- azhar university, cairo, Egypt.

'self' from what is foreign (Jeff, 2015). Squalene had undergone toxicity effectors in the biological pathways including immunopathological effects (Eid*et al.,* 2015). In spite of spleen is one of the centers of activity of the reticuloendothelial system and can be considered analogous to a large lymph node (Brender, 2005). It also can be affected by adjuvant action (Eid*et al.,* 2015). Spleen is the body's largest secondary lymphoid organ with important roles in regard to red blood cells and the immune system (Thanya*et al.,* 2016). It synthesizes antibodies in its white pulp and it has been found to contain in its reserve half of the body's monocytes within the red pulps. These monocytes, upon moving to injured tissue, turn into dendritic cells and macrophages (Jia and Pamer, 2009).Squalene as adjuvant caused many pathological changes in splenic tissue by losing its architectureincrease proliferation of the white pulps with highly elongated, branched and thickened wall of the central arterieswith highly disturbed white pulps (Eid*et al.,* 2015). Adjuvants resulting intercellular and humoral responses leading to autoimmunity and lymphoma development, chronic stimulation of the immune system is thought to be the key mechanism through which autoimmune diseases can lead to lymphomagenesis. Many adjuvants can act similarly perturbing immune system's function, inducing a state of prolonged immune activation related to chronic lymphatic drainage. Several mechanisms were proposed by which adjuvants induce inflammation. Some of them are triggering inflammasome; others bind DNA, lipid moieties in cells. The sustained inflammation increases the risk of genetic aberrations, where the initial polyclonal activation ends in monoclonality. The latter is the hallmark of malignant lymphoma. Thus, chronic adjuvant stimulation may lead to lymphoma (Dana andYehuda, 2015). A single intradermal injection of the adjuvant-oil squalene induced stimulation of lymphoid cell proliferation (Holm *et al.,* 2004). Immunohistochemistry (or IHC) is a method for demonstrating the presence and location of proteins in tissue sections. It enables the observation of processes in the context of intact tissue. This is especially useful for assessing the progression and treatment of diseases. In general, the information gained from IHC combined with microscopy literally provides a "big picture" that can help make sense of data obtained using other methods(Buchwalow and Böcker, 2010). Immunohistochemical staining is accomplished with antibodies that recognize the target protein. Since antibodies are highly specific, the antibody will bind only to the protein of interest in the tissue section. The antibody-antigen interaction is then visualized using chromogenic detection, in which an enzyme conjugated to the antibody cleaves a substrate to produce a colored precipitate at the location of the protein (Oliver *et al.,* 2010).Proliferating cell nuclear antigen (PCNA) is identified as the polymerase-associated protein that is essential for replication and also known as cyclin. PCNA acts as a homotrimer and achieves its processivity by encircling the DNA, where it acts as a scaffold to recruit proteins involved in DNA replication, DNA repair, chromatin remodeling and epigenetics (Moldovan *et al.,* 2007). Many proteins interact with PCNA via the two known PCNA-interacting motifs PCNA-interacting peptide (PIP) box (Warbrick, 1998) and AlkB homologue 2 PCNA interacting motif (APIM) (Gilljam*et al.,* 2009).It has been proposed that the mechanism wherebyadjuvantedvaccines induce autoimmune responses can be defined as the possibility that amino acid sequence similarities between foreign and self-peptides are sufficient to elicit cross activation of autoreactive T or B cells by pathogen-derived peptides (Kohm*et al.,* 2003). Limited informations are available on the relative toxicityofsqualene as adjuvant on different tissues. The damage was done by squaleneadjuvanted vaccine, particularly occurred when it potentiated by powerful "immunoenhancers" caused a strong immunostimulation that causes some histopathological effects (Eid*et al.,* 2015). Due to many pathologic changes that occurred after squalene immunization, the present study was planned to determine the immune activation and the histopathological changes in the spleen tissue of Albino rats.

Material and methods:-

Animals:-

Young Albino rats weighing 65-70 gm were obtained from The Nile Company for Pharmaceutical and Chemical Industries. After an acclimatization period of one week, the animals were housed in bottomed cages in a room under standard conditions. They were provided with water and a balanced diet. All animals received care in compliance with the Egyptian rules for animal protection.

Study design:-

Animals were divided into different treatment groups as follows: the first group (Con), the second group received intramuscular injection of squalene adjuvant (Sq1, AS03) at the zero day, the third group received second intramuscular injection dose at the 21st day (Sq2, AS03) and the fourth groupserved as long period vaccination effect (SqL, AS03). Animals of all the treated groups vaccinated with 0.125ml, the same dose of a human multiplied by conversation factor (0.018)/200 gm of body weight of rat according to Paget and Barnes (1964). Animals of all groups, Con, Sq1, Sq2 and SqL were decapitated after 10, 30 and75days post-stimulation (n= 10). The collected serum was assayed for immunological parameters and lymph nodes of rats were carefully removed and preparedto use for the various histopathological and histochemical determinations.

Immunohistochemical studies:-

Expression of proliferating cell nuclear antigen (PCNA) or cyclin or polymerase delta auxiliary protein is elevated in the nucleus during late G1 phase immediately before the onset of DNA synthesis, becoming maximal during S-phase and declining during G2 and M phases. Its level correlates directly with rates of cellular proliferation and DNA synthesis. PCNA/cyclin may act as an auxiliary protein of DNA polymerase-delta to play a fundamental role in the initiation of cell proliferation.Immunoprecipitation occurred as antibody purification from ascites fluid by Protein A, that isprepared in 10mM PBS. And it is ready-to immunohistology technique (Formalin/paraffin), the staining of tissues that have been fixed (in neutral buffered formalin) and thenembedded in paraffin before being sectioned.

Expression of proliferating cell nuclear antigen in spleen tissue was determined according to Jain *et al.* (1991) using Proliferating Cell Nuclear Antigen (PCNA) Ab-1 (Clone PC10) ImmunohistochemicalStain kit; it act as an auxiliary protein of DNA polymerase-delta to play afundamental role in the initiation of cell proliferation, Thermo Fisher Scientific, (Cat. #MS-106-R7).

Histological examinations of the spleen:-

Spleen tissues of immunized rats were washed in saline and fixed in 10% neutral formalin, embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin stain according to the method of Drury and Wallington (1980) and Mallory's trichrome stain according toPearse(1977) for light microscopic observations.

Histochemical examinations of the spleen:-

Spleen tissues of immunized rats were prepared and stained with periodic acid Schiff technique according to Pearse (1977) and mercuric bromophenol blue according toMazia*et al.*(1953) for light microscopic observations**.**

Results:-

Immunohistochemical changes in rat spleen tissue:-

The severity comparative evaluation of immunoreaction in spleen tissue obtained from non-immunized control group, squalene- immunized groups after the first (Sq1), the second (Sq2) dose and long period effect (SqL) of this study after 10, 30 and 75 days of treatment respectively using proliferating cell nuclear antigen are shown in Table 1. Negative immunoreactions were observed in splenic tissue of a rat of the control group stained with proliferating cell nuclear antigen (Fig. 1A, B). While splenic tissue of a rat of the squalene treated group after the first dose (Sq1) showing moderate positive immunoreaction in the white pulps and numerous moderately stained lymphocytes scattered in the red pulps (Fig. 2A, B, C). A dense positive immunoreaction in the white pulps and scattered stained lymphocytes outside it were observed in splenic tissue of rats of the squalene treated group after the second dose (Sq2), but degenerated lymphocytes showed negative staining affinity, with highly dilated and elongated trabecular vein which contains negatively stained hemolysed blood cells (Fig. 3.A, B, C, D). The splenic tissue of rats of the squalene long period group (SqL) stained with proliferating cell nuclear antigen showing a slight immunoreaction within and outside the white pulps (Fig. 4.A, B).

Table1: -Comparative evaluation of immunoreaction in spleen tissue.

Groups **Immuno reaction**	**Control**	**Sq1**	**Sq2**	**Sq (L)**
proliferating cell nuclear antigen (PCNA)	-ve	++ ve	+++ ve	+ ve
Figure no.	Fig.1	Fig. 2	Fig. 3	Fig.4

Sq1= squalene- immunized groups after the first, Sq2 = squalene- immunized groups after the second dose, SqL= squalene long period effect after 10, 30 and 75 days of treatment, - ve= nil immunoreaction, + ve = mild immunoreaction, ++ ve = moderate immunoreaction, +++ ve =severe immunoreaction (n= 10rats in each group).

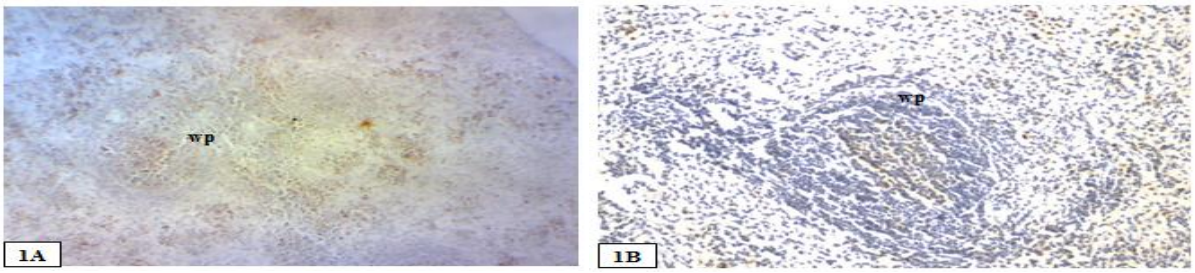

Fig 1:- A&B:- Splenic tissue of a rat of the control group stained with proliferating cell nuclear antigen (1A X 50& 1B X 200).

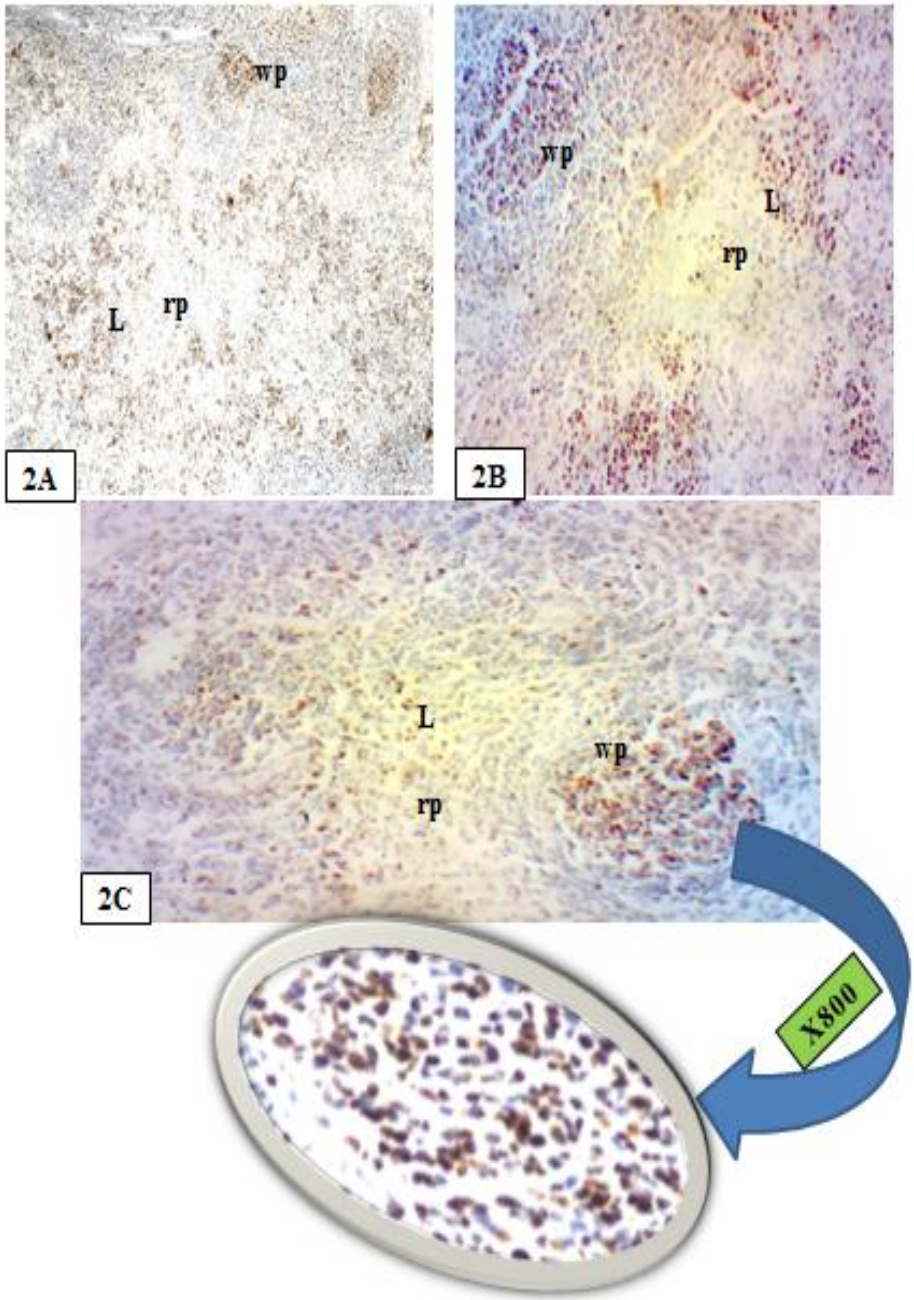

Fig 2:- A, B &C:- Splenic tissue of a rat of the squalene treated group after the first dose (Sq1) stained with proliferating cell nuclear antigen (2A X 50, 2B&C X 200).

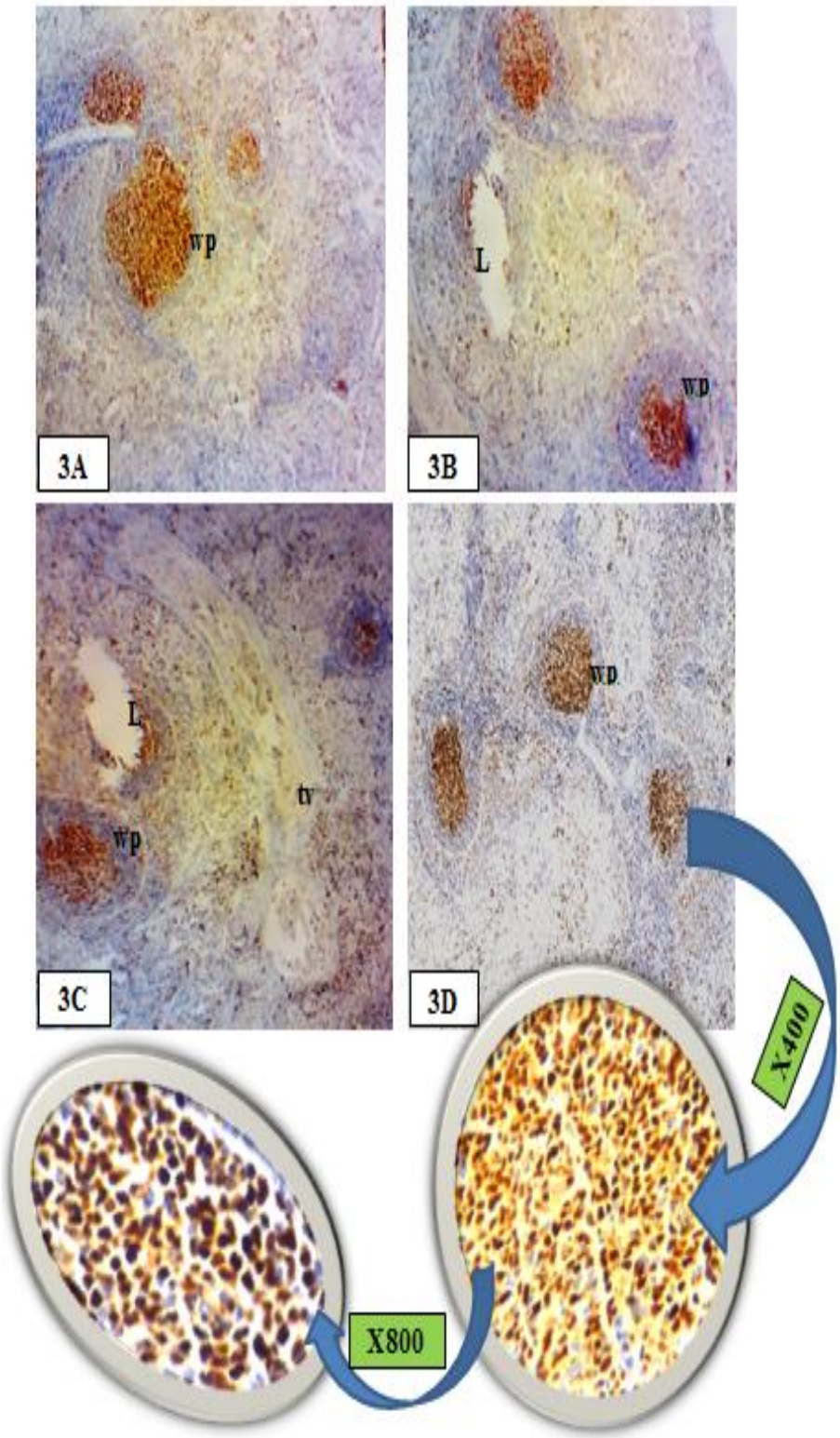

Fig 3:- A, B, C& D:- Splenic tissue of a rat of the squalene treated group after the second dose (Sq2) stained with proliferating cell nuclear antigen showing (3A, B, C & D X 100).

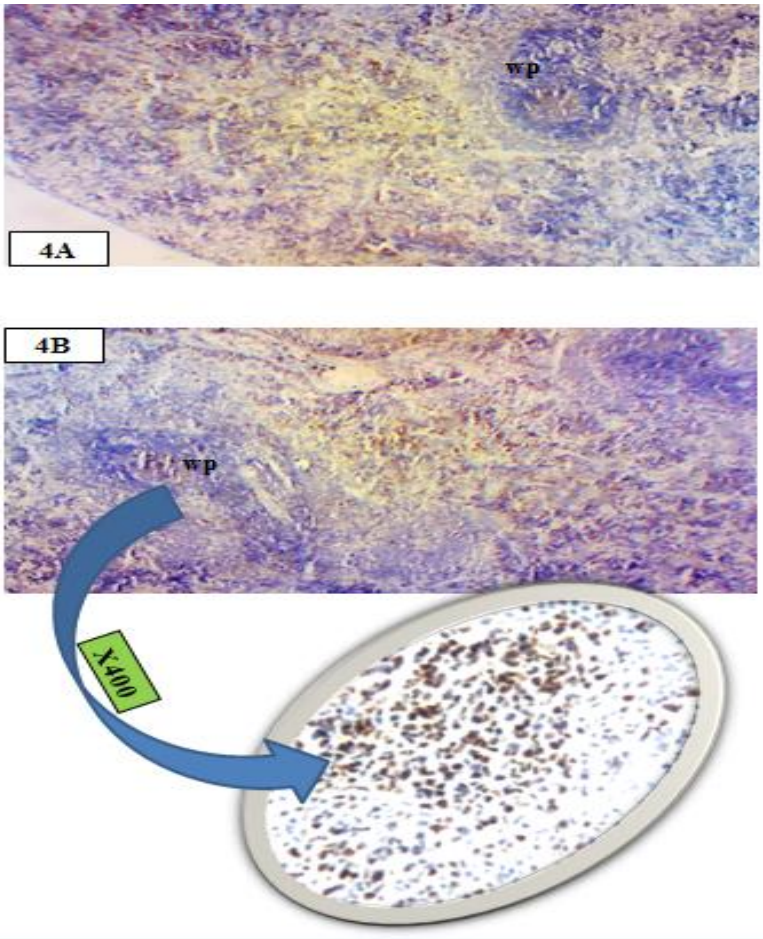

Fig 4:- A&B:- Splenic tissue of a rat of the squalene long period group (SqL) stained with proliferating cell nuclear antigen (4A&B X 100).

Histopathological changes in rat spleen tissue:-

Representative sections of the spleen are shown in Fig. 5A–D and Fig. 6A–D. Splenic tissue sections from a rat of the control group stained with hematoxylin and eosin showed normal white pulp with the central arteries, trabecula and red pulp (Fig. 5.A), while squalene treated group after the first dose (Sq1) showing that increased number of connected white pulps, increased proliferation of lymphocytes in them, thickened trabeculae with highly dilated and elongated trabecular vein which contained hemolysed blood cells. Notice: highly thickened and elongated walls of the central arteries with narrow lumen, delaminated, ruptured and distorted capsule, degenerated areas, numerous necrotic areas, distorted white pulps, numerous hemosidrin granules, pools of hemolysed RBCs and highly widened and elongated splenic vein, artery (Fig. 5.B1, 2, 3), squalene treated group after the second dose (Sq2) showed increased proliferation of WBCs with bizarre arrangement of them in the white pulps, highly elongated, destructed and thickened walls of the central arteries, thickened trabeculae, numerous necrotic areas, lots of degenerated areas. Notice: pools of degenerated RBCs with numerous hemosidrin granules (Fig. 5.C1, 2), in addition to increased number of connected white pulps that observed in squalenelong period effect group (SqL), while others appeared somewhat normal and thickened walls of some central arteries,but red pulps show normal architecture with dilated and elongated trabecular vein (Fig. 5.D1, 2). The rat splenic tissue of the control group stained with Mallory's trichrome stain showed thin collagen bundles supporting the capsule, red pulps and white pulps (Fig. 6.A), squalene treated group after the first dose (Sq1) also showed increased collagen bundles especially under the capsule, in the trabeculae, wall of the blood vessels, through red and white pulps notice, hemolysed blood cells inside the blood vessels with red brightly stained pools of RBCs (Fig. 6.B1, 2, 3), while after the second dose (Sq2) highly increased collagen bundles in the splenic tissue especially around the splenic vein, in and under the capsule and in the red and white pulps were observed (Fig. 6.C1, 2), otherwise squalenelong period effect group (SqL) showed deeply stained hemosidrin granules and increased collagen bundles in the splenic tissue especiallyin the white pulps (Fig. 6.D).

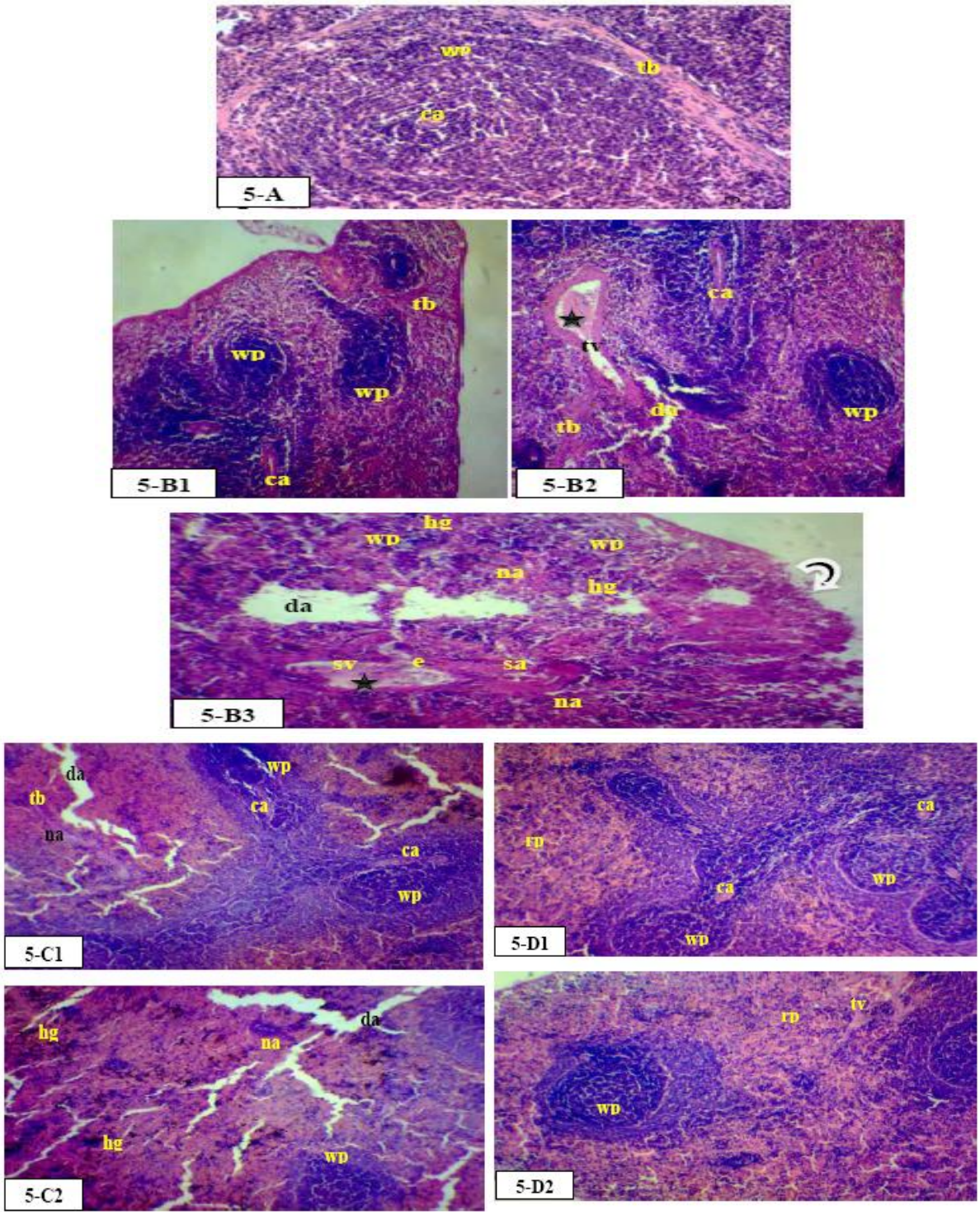

Fig 5:- Representative images of histopathological changes in rat splenic tissue (H&E, original magnification x 100,B2X200) (A–D) (n = 10 rats in each group). **(A)** Control group: wp—white pulp, ca —central arteries, tb—trabecular, rp— red pulp. **(B1, 2, 3)**Squalene treated group after the first dose (Sq1): wp— white pulps, tb —trabeculae, tv— trabecular vein, * —hemolysed blood cells, ca —central arteries, ↑ —capsule, da —degenerated areas, na —necrotic areas, hg —hemosidrin granules, sv —splenic vein, sa —splenic artery.**(C1, 2)**Squalene treated group after the second dose (Sq2): wp —white pulps, ca —central arteries, tb —trabeculae, na —necrotic areas, da —degenerated areas, hg— hemosidrin granules. **(D1, 2)**Squalenelong period effect group (SqL): wp—white pulps, ca —central arteries, rp— red pulps, tv —trabecular vein.

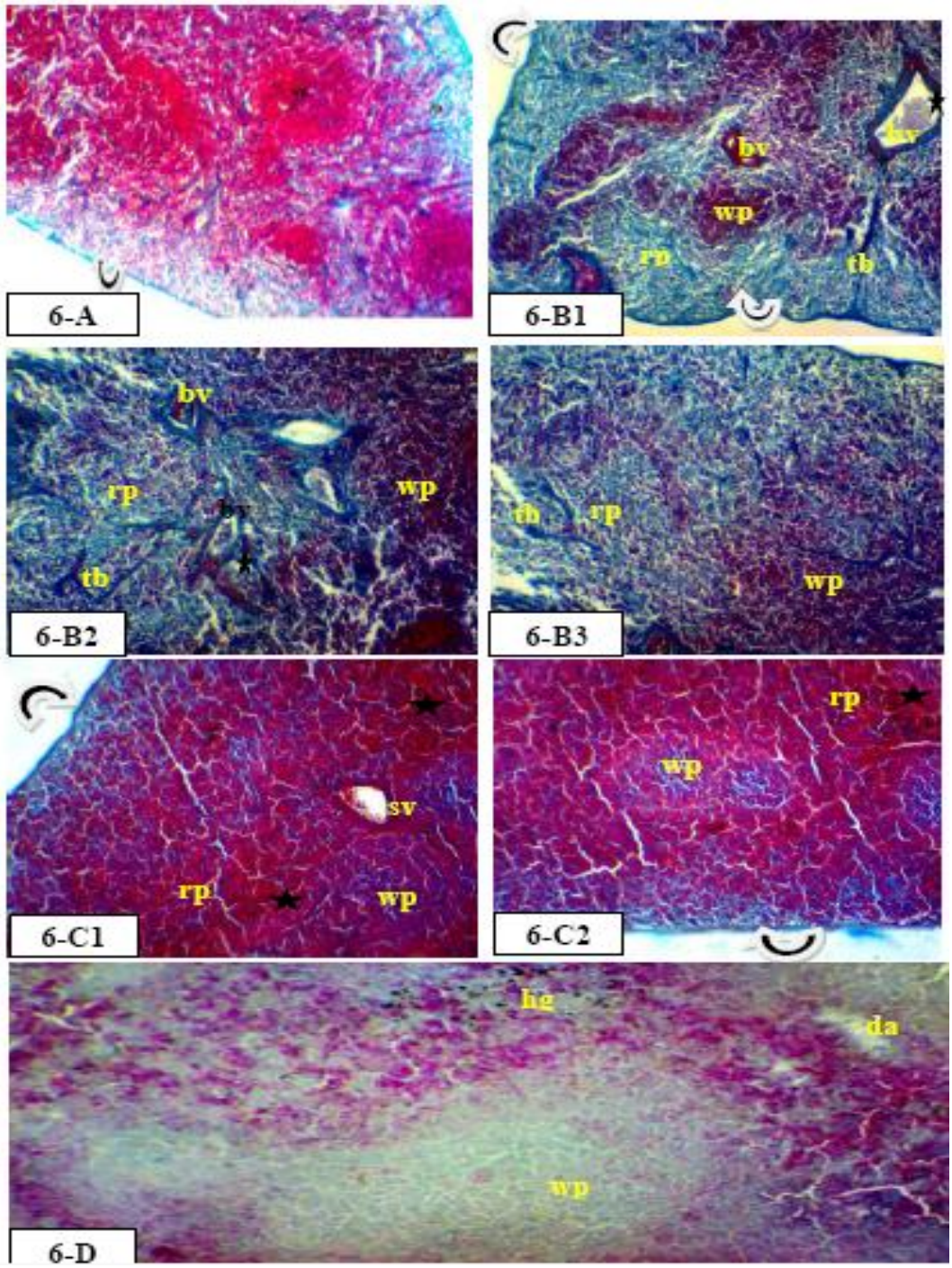

Fig 6:- Representative images of histopathological changes in rat splenic tissue (Mallory's trichrome stain, original magnification: A, C x 50, B, D x 100) (A–D) (n = 10 rats in each group). **(A)** Control group: ↑ —capsule, wp—white pulp, rp— red pulp, **(B1, 2, 3)**Squalene treated group after the first dose (Sq1): ↑ —capsule, wp— white pulps, rp— red pulp, tb —trabeculae, bv— blood vessels, * —hemolysed blood cells,**(C1, 2)**Squalene treated group after the second dose (Sq2): sv —splenic vein,↑ —capsule, rp— red pulp, wp —white pulps, * — pools of RBCs, **(D1, 2)**Squalenelong period effect group (SqL): wp— white pulps, hg —hemosidrin granules.

Histochemical changes in rat spleen tissue:-

Results of histochemical changes in the spleen are shown in Fig. 7A–D and Fig. 8A–D. Splenic tissue of rats of the control group stained with periodic acid Schiff's reaction (PAS) showed normal distribution of PAS positive materials in the splenic tissue notice, deeply stained walls of the central arteries (Fig. 7.A), squalene treated group after the first dose (Sq1) showed highly thickened and deeply stained walls of the splenic vein, artery, trabeculea, walls of central arteries with reduced staining affinity in the red and white pulps and negatively stained degenerated areas,with lots of hemosidrin granules, in addition, deeply stained walls of the blood vessels which contain hemolysed RBCs (Fig. 7.B1, 2), while squalene treated group after the second dose (Sq2) showed moderately stained PAS +ve materials in the white pulps with diffused staining affinity in the red pulps and numerous hemosidrin granules, negatively stained degenerated areas, highly thickened and deeply stained trabeculae and walls of the blood vessels which containing hemolysed RBCs (Fig. 7.C1, 2). A normal staining affinity of PAS +ve materials in the white pulps was observed in squalenelong period effect group (SqL) with highly decreased staining affinity in the red pulps, in spite of the trabeculae and walls of the thickened central arteries show dense staining affinity, while hemolysed blood cells inside the highly dilated trabecular vein are moderately stained (Fig. 7.D). Spleen tissue of rats of the control group stained with mercuric bromophenol blue showed normal distribution of total protein in the splenic tissue (Fig. 8.A), squalene treated group after the first dose ,Sq1) showed highly decreased total protein in the splenic tissue with negatively stained degenerated areas and reduced staining affinity in and under the capsule , in the red and white pulps, notice: deeply stained hemosidrin granules in the red pulps and moderately stained trabeculae and walls of the blood vessels (Fig. 8.B1, 2). A highly decreased total protein in the splenic tissue with negatively stained degenerated areas and deeply stained hemosidrin granules were noticed in squalene treated group after the second dose (Sq2) (Fig. 8.C1, 2). Squalenelong period effect group (SqL) showed highly decreased total protein in the splenic tissue with negatively stained degenerated areas and deeply stained capsule, trabeculae and walls of the central arteries (Fig. 8.D).

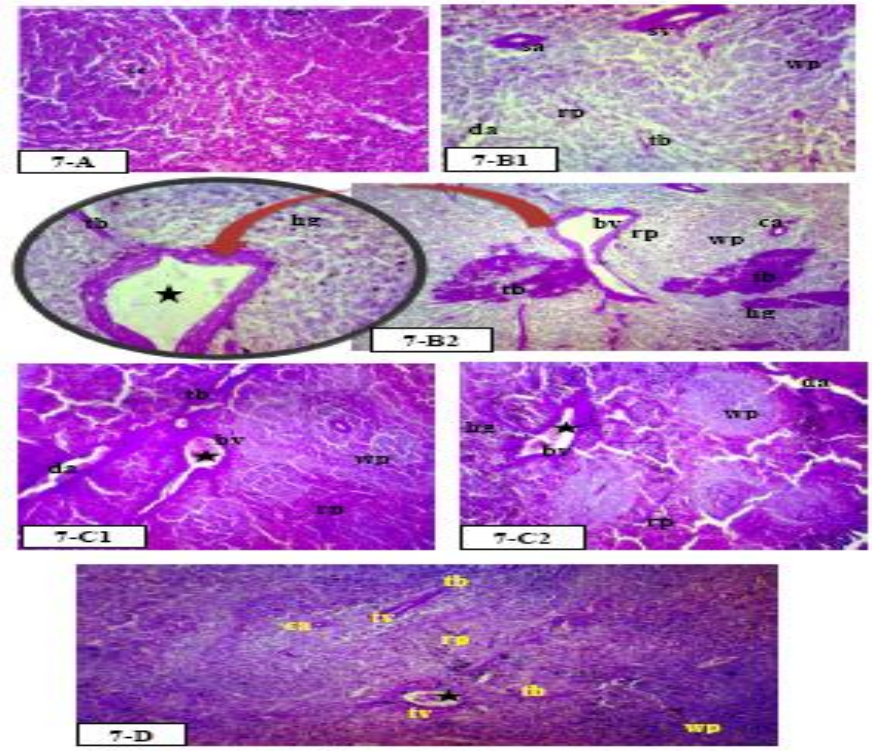

Fig 7:- Representative images of histochemical changes in rat splenic tissue (periodic acid Schiff's reagent, original magnification x 100, B2 x 200) (A–D),n = 10 rats in each group). **(A)** Control group: ca —central arteries, **(B1, 2)**Squalene treated group after the first dose (Sq1): sv— splenic vein, sa —splenic artery, tb —trabeculea, ca —central arteries, rp —red pulps, wp — white pulps, da —degenerated areas, hg —hemosidrin granules, bv — blood vessels, * —hemolysed RBCs, **(C1, 2)**Squalene treated group after the second dose (Sq2): wp —white pulps, rp —red pulps, hg —hemosidrin granules, da —degenerated areas, tb —trabeculae, bv— blood vessels, *— hemolysed RBCs, **(D)**Squalenelong period effect group (SqL): wp —white pulps, rp —red pulps, tb —trabeculae, ca —central arteries, *—hemolysed blood cells, tv —trabecular vein.

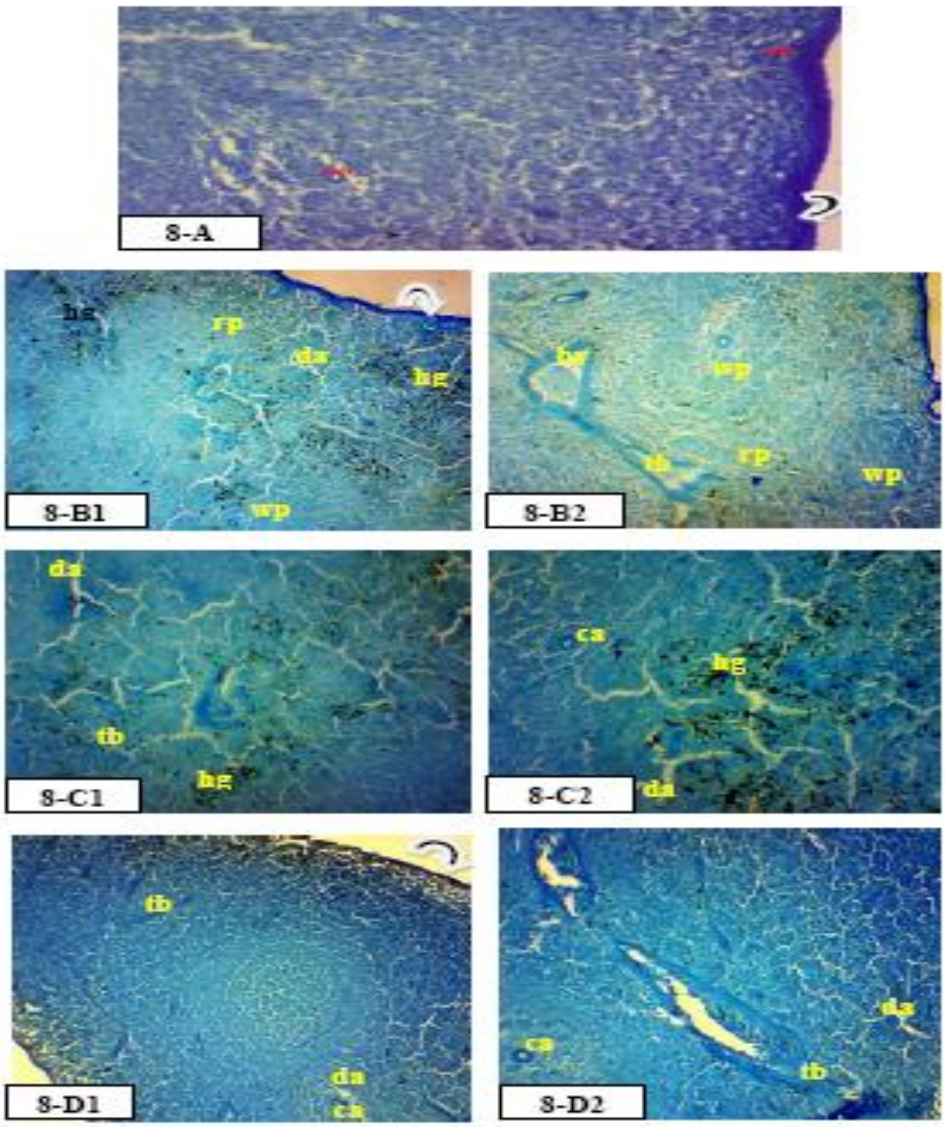

Fig 8:- Representative images of histochemical changes in rat splenic tissue (mercuric bromophenol blue, original magnification x 100) (A–D) (n = 10 rats in each group). **(A)** Control group: ↑ —capsule, tb—trabeculae, ca—central arteries, **(B1, 2)**Squalene treated group after the first dose (Sq1): da— degenerated areas, ↑—capsule, rp—red pulps, wp—white pulps, hg—hemosidrin granules, tb—trabeculae, bv—blood vessels, **(C1, 2)**Squalene treated group after the second dose (Sq2): da —degenerated areas, hg— hemosidrin granules, tb— trabeculae, ca— central arteries, **(D)**Squalenelong period effect group (SqL): da— degenerated areas, ↑— capsule, tb— trabeculae, ca— central arteries.

Discussion:-

In the present study the severity comparative evaluation of immunoreaction in spleen tissue using proliferating cell nuclear antigen (PCNA) showed moderate immunoreaction in squalene- immunized group after the first dose, severe immunoreaction in the second dose and mild immunoreaction in long period effect. These resultsare in agreement with those of Kedl and Kedl (2015) who stated that squalene enhances the magnitude and quality of adaptive immune responses.Squalene oil-in-water emulsions elicit both cellular (Th1) and humoral (Th2) immune responses, this class of adjuvants is believed to act through recruitment and activation of antigen presenting cell (APC) and stimulation of cytokines and chemokines production by macrophages and granulocytes (Calabro*et al.*, 2013). In addition adjuvants are broadly defined as carrier/delivery systems that provide means to enhance the durability and strength of the adaptive immunity, as well as opportunities to steer immune responses toward antibody and/or T-cell-based immunity for optimal protection against a specific disease (Smed-Sorensen and Lore, 2013). In contrast, a

higher lymphocyte proliferation assay (LPA) indexes were found at week 12 after intramuscularlyadministeredsqualeneadjuvanted vaccine (Colombatto*et al.,* 2014).Injection of the synthetic adjuvant formulated in a squalene oil-in-water emulsion (SE)leading to antigen-independent activation of lymphocytes (Matloubian*et al.,* 2004). This adjuvant capable of inducing robust B cell that operates through activation of lymph node (LN) macrophages (Orr *et al.,* 2016).

In the present study spleen of squalene treated group after the first dose showed many drastic changes in the white pulps. These changes include increased number of connected white pulps with increased proliferation of lymphocytes.Dilated and elongated trabecular veins which contained hemolysed blood cells with ruptured endothelial lining and thickened trabeculea, numerous degenerated areas, necrotic areas and numerous of haemosidrin granules are also noticed. While after the second dose splenic tissues showed bizarre structure, but long period effect group appeared somewhat normal. Which are in agreement with those of Eid*et al.* (2015). Adjuvant oil exposure triggers both systemic acute phase reactions and local activation of the peripheral lymphoid system (Svelander*et al.,* 2001), in addition adjuvant-mediated enhancement of cellular activation, antigen uptake and accumulation of antigen^{+} cells in the tissue and subsequently in the draining lymph nodes(dLNs)wich are essential immune events preparing for antigen presentation and generation of antigen-specific immunity (Ghimire*et al.,* 2012). As neutrophils represent one of the antigen+ cell types in the dLNs, evaluation of their role in stimulation of the adaptive immunity is warranted, splenic neutrophils supported antibody responses via cytokines (Puga*et al.,* 2012).

Squalene exhibited a significant increase in relative spleen weights of the mice and splenic plaque forming cells (Young and Joung, 1991). According to Viera (2001), Harold (2005) and Anthony (2009) adjuvants are foreign to the body and cause adverse reactions, they have toxic effect at the molecular level and cause a morphological changes, including membrane alterations and cell shrinkage. Adjuvant mediated cell death as toxic effects (Humphrey *et al.,* 2005). The toxicity-related elevation of micronuclei within the primary human lymphocytes, this genotoxic effects were seen even at concentrations which can occur at the injection site (Westphal*et al.,* 2003). Adjuvant alsogenerates cytotoxic T lymphocytes which are able to lyse target cells (Anthony and Allison, 2002) and cause little tissue reaction as liposomes and immune-stimulating complexes (ISCOMs1) (Gomez-Vargas *et al.* 2004).

The progressive dilation and congestion of blood sinuses observed in the present study were also detected by Eid*et al.* (2015) and can be explained by Ellershawand Gurney (2001) who reported that progressive dilation and congestion of blood sinusoids could be considered as a reactive change that may be related to the inhibitory effect of inositol trisphosphate (IP3) on the vascular smooth muscles which induced relaxation and consequent. This vasodilatation and increased vascular permeability may lead to loss of fluid from the blood, so the vessels are engorged with blood cells with consequent slowing down of the blood stream which would result in degeneration and necrosis in the tissues.This may be considered as a reaction to progressive epithelial cell death and atrophy of the tissuewhich could be related to adjuvant effects.Vaccinated unadjuvanted groups showed a clear pattern of dead cells rather than significant increase in cell death observed in adjuvanted group, these findings suggest that adjuvant evokes further cell membrane damage, resulting in more localized cell death post immunization which could lead to higher immune response Depelsenaire*et al.* (2014) and also this investigation emphasized byShen and Yang (2015) who stated that *in vitro* cell death associated with oil-water emulsions adjuvants models consumption.

In the present study squalene as adjuvant may also exhibit impaired endothelial responses in regions that are more prone to the detrimental effects of disturbed flow and thus have increased atherosclerosis. This observation is in agreement with those of Natalie *et al.* (2001) who stated that endothelial damage at a site predisposed to atherosclerosis in association with the disturbed flow characteristic of that site. In addition,squalene is hypolipidemic by mediated mechanism that regulates the expression of lipid metabolism genes in hepatocytes (Hoang *et al.,* 2016). Hyperlipidemia is a medical disorder characterized by elevated levels of lipids including cholesterol and triacylglycerols in the blood that can lead to the progression of atherosclerosis (Nelson, 2013).

Results of the present study showed many histopathological alternations such as degenerated areas and hemosidrin granules under the effect of squalene on the cytoskeleton. Nancy *et al.* (2004) and Joseph (2013) concluded that the inflammatory response observed in rats with adjuvant-induced arthritis was accompanied by significant biochemical changes of plasma acid phosphatase and membrane-bound neuraminidase activity after adjuvant injection. The possible role of neuraminidase in the degradation of tissue components and in the development of the

immunological features as rheumatoid arthritis, Nunia*et al.* (2007) reported that rupture of number of the red blood cells membrane caused out leakage of hemoglobin and hemosidrin.

As well as adjuvant increase vascular permeability that caused local tissue destruction and result in systemic effects that include fever (Harold, 2005). Increased permeability of cell membrane caused osmotic swelling leading to erythrocyte hemolysis (El-Beih*et al.,* 1995). These results declare the hemolysis observed in the present study.
Many necrotic and degenerated areas were observed in tissues of lymphatic organs in the present research all over the experimental periods, however the damage severity was observed after the second dose treatment. These results were explained by Burk *et al.* (1995) who attributed necrosis to the depletion of glutathione in the tissue. But Orr and Blakley (1997) suggested that such necrotic lesions may be due to either a progressive degenerative action of intracellular enzymes of the injured cells or to the metabolic disturbance and inhibition of protein synthesis in the tissue. Instead Leek *et al.* (1999) attributed necrosis to insufficient supply of the blood to an area of the tissue.While Khaki *et al.* (2006) reported that decreased ribosomes, glycogen granules and cristae of mitochondria may lead to corrugatedmembranes. Cytochrome c was shown to leak from the mitochondria and cells undergoing apoptosis and necrosis, so adjuvant mediated cell death as toxic effects (Humphrey *et al.,* 2005).

Results of the present study showed increased stain affinity of collagen fibres in splenic tissue of all the treated groups, especially in the thickened capsule, trabeculea, walls of blood vessels and red and white pulps, with brightly stained pools of RBCs after the second dose. These changes in agreement with those of Eid*et al.* (2015) who reported that lymphatic tissues exhibited highly elevated collagen fibres after adjuvanted and non- adjuvanted vaccine treatment and this may be explained by the detrimental effect on the tissue and endothelium (Natalie *et al.,* 2001) which occurred by squalene immunization and this result come in the same line with those of Hassan *et al.* (1988) who recorded that increased collagen fibres may lead to increase the defense reaction against toxic materials. In addition, vascular bleeding can be a challenge, especially when it stems from a vein embedded in the fatty tissue(Selman and Latifi, 2016).

Also collagen elevation may be due to squalene- related to arthritis, this suggestion comes in the same line with those of Kakizoe*et al.* (1999) who found that arthritis was induced by immunization with intradermally emulsion made with adjuvant.Immunized group showed collagen bundles accumulated around the destructed joints in accordance with the pathological findings as fibroproliferative disorder associated with type II collagen (CII)-induced arthritisthat consider ones of vaccine causes. William *et al.* (2012) stated that arthritis is a type of collagen vascular diseases that associated with collagen and blood vessel abnormalities and autoimmune in nature and it undergoes autoimmune connective tissue disorders. While Zhang *et al.* (2006) declared that increased collagen may lead to rapid healing, rapid differentiation of cells and appearance of a new network of blood vessels. But Rousovan*et al.* (1992) stated that the increase in collagen fibres may be due to increased interstitial tissue and the white fibres. Horn *et al.* (1985) reported that the presence of collagen in the presinusoidal spaces might affect the blood supply to cells and would reduce the exchange of metabolites, perhaps causing cellular dysfunction and necrosis.
Decreased PAS positive materials were detected in red and white pulps of splenic tissue in this study, but highly thickened and deeply stained trabeculea and blood vessels containing hemolysed RBCs were realized with numerous hemosidrin granules in addition to negatively stained degenerated areas after the first and the second doses, while normal staining affinity of PAS +ve materials was realized in the white pulps and decreased stain affinity in the red pulps were detected in long period effect group. Decreased glycogen content observed in the tissue post vaccination may be due to vacuolation and degeneration of the tissue that occurred by action of squalene, this observation comes in the same line with those of Eid*et al.* (2015). The adjuvant may incorporate carbohydrate moieties or molecular configurations that increase delivery to macrophages and dendritic cells via specific receptors (Bonifaz*et al.,* 2004), also adjuvant acts as immunomodulator by recruitment and activation of macrophages and dendritic cells which lead to other inflammatory sequelae (Harold, 2005).
Increased staining affinity of RBCs inside the blood vessels was discussed by Junqueira and Carneiro (2003) who stated that RBCs contain 10% carbohydrates of their weights, this may explain increased staining affinity of PAS+ve materials inside the congested sinuses and hemorrhagic areas observed in this study.
Highly reduced total protein was detected in the splenic tissue with negatively stained degenerated areas and deeply stained hemosidrin granules in all the treated groups, while, in long period effect group deeply stained capsule and trabeculea were observed.

This protein depletion may be due to the ability of squalene as adjuvant to induce lysis of target cells by initiation of mitochondrial reaction (Anthony and Allison, 2002), or tissue reaction as liposomes stimulating complexes (Gomez-Vargas *et al.,* 2004). It may also result by tissue destruction as activated vascular permeability and result in systemic effects that include fever and the production of acute-phase proteins (Harold, 2005). Meanwhile Gorczynska (1987) and Al Gahtani (2006) reported that vacoulation and degeneration led to decreased protein content in the tissue, but congested blood vessels and hemorrhagic areas showed increased RBCs which was accompanied by increased stain affinity of total protein.

Decreased protein content noticed in the present study was also realized under physical and chemical factors that also emphasized the previous observations by El Banhawy*et al.* (1986) who noticed that decreased protein content may be due to increased action of lytic enzymes. In 2007, Eid and Al Dossary stated that decreased protein content in tissue may be due to the drastic effect on the rough endoplasmic reticulum (RER), mitochondria and Golgi apparatus and increased lysosomes in the cells.

Conclusions;-

Our data reveal that squalene as adjuvant that used in many vaccines showed a higher immune potential after two doses than one only. Despite the adverse events induced post administration of squalene that showed slowly recovery after long period effect. Squalene as adjuvant showed a drastic immunoological changes in addition to the sever immunohistological and histopathological changes.

Acknowledgement:-

The authors are thankful to Dr. Aly F. Mohamed, Prof. of Virology, Applied research manager, The Holding Company for Biological Products and Vaccines (VACSERA) and Department of Zoology, Faculty of Science (Girls branch), Al- Azhar University for providing laboratory facilities and technical assistance.

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