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Evaluation the Effectiveness of Green Zinc Oxide Nanoparticles on The Anti-Inflammatory Effect of Dexamethasone and Its Side Effects in Rats

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Abstract

Green synthesis of nanoparticles is considered an ecofriendly technology because it does not involve toxic chemicals. In this study, green zinc oxide nanoparticles (7-25 nm) were synthesized from olive plant and examined for its anti-inflammatory activities alone or with dexamethasone in rats. Seventy rats were randomly allocated into 7 groups (10 rats/group). Group 1 served as a negative control. Other rats in the six groups were injected with formalin into the intra planter paws of right hind limb (50ul) followed by administration of investigated agents as following; group 2 used as control positive for inflammation. Group 3 and 4 were administrated dexamethasone at 2 doses; 2 and 5mg/ kg b.wt. (i.m), respectively. Group 5 was treated with zinc oxide nanoparticles at dose of 25mg/ kg b.wt.(i.p). Groups 6 and 7 were treated with Zinc oxide nanoparticles at dose of 25 mg/kg b. wt. (i.p) and dexamethasone at doses of 2 and 5mg/kg b. wt. (i.m), respectively. All treatments were continuous for 5 successive days. Representative blood and tissue samples for immunological, chemical and pathological investigations were collected. The present study refers to the anti -inflammatory effect of both zinc oxide nanoparticles and dexamethasone through variable pathways; mixed treatment between green Zinc oxide nanoparticles and dexamethsone improve the dysregulated biochemical effects induced by inflammation. ZnO nano-particles affect mainly Th2 which enhanced by dexamethasone that had mainly immune- suppressive effect on B-lymphocytes. Despite the recorded anti-inflammatory effect, attention to side effects such as genotoxicity recorded in that study, should be considered. We could conclude that although green zinc oxide nanoparticles may be considered as a potential treatment for inflammatory conditions, but it enhances the apoptotic effect of dexamethasone with end-result of (transient) genotoxic effect.

Keywords: Green Zinc Oxide, Nanoparticles, Dexamethasone, Inflammation, Rats.

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Introduction

Metal nanoparticles (NPs) and their oxides have a considerable number of present and future applications in the medical and industrial fields. The smaller size, unique physicochemical properties and surface modifications of the NPs has substantially improved the application of them (Mody et al., 2010). Nanoparticles are more highly absorbed into the respiratory, skin and gastrointestinal systems than micron-sized particles (Fubini et al., 2010).

Green synthesis of nanoparticles is one of the most recommended biological methods for the synthesis of nanoparticles through utilizing of plants that contain natural reducing agents such as flavonoids (Myriam et al., 2012). Zinc (Zn) is typically the second most abundant transition metal in organisms after iron and it is one of the essential trace elements that play important roles including; nutrients metabolism, growth and development of bone. Zinc was established to be essential for normal functioning of the immune system through its mediating effect on T-lymphocytes (Prasad and Elumalai, 2011). Zinc also has a role in the synthesis and/or breaking down of carbohydrates, lipids, proteins, and nucleic acids, in addition to its antioxidant effect (Ahmadi et al., 2014). Multiple studies have revealed that zinc supplementation has anti-edematous activity, especially in the early phase of inflammatory response, and increases anti-inflammatory activity of non-steroid anti- inflammatory drugs (Gawel et al., 2014). Inflammation is the first response of the immune system that helps the body to protect itself against injurious stimuli. It can be evoked by a variety of noxious agents (e.g. chemical agent, infections, antibodies or physical injuries). Inflammation may be acute or chronic reaction which associated with different reaction including humoral and cellular process (Saha and Ahmed, 2009). Dexamethasone is a potent and highly selective synthetic glucocorticoid with a relevant clinical use mainly due to its anti-inflammatory and immunosuppressive effects (Abbas and Saeid, 2015).

The aim of the present study was to evaluate the effect of green zinc oxide nanoparticles as anti-inflammatory agent alone and /or with dexamethasone to reduce the possible hazardous effect of glucocorticoids.

Materials and methods

I -Plant and chemicals

Green Olive plant (*Olea europeae*) leaves were collected from olive farm. All the chemicals used in the extraction process were obtained from sigma-Aldrich chemicals, Egypt.

Synthesis of green zinc oxide nanoparticles:

Synthesis of green ZnO (NPs) was based on the biosynthesis method (Nagajyothi et al., 2013) with some modifications. Briefly; olive plant green leaves were washed with sterile distilled water, air dried, cut into small pieces and mixed with 250 ml of boiled deionized water. Zinc nitrate was dissolved in distilled water under constant stirring using magnetic stirrer

and added after complete dissolution to olive Plant green leaves extract. The mixture was kept under stirring at 90°C for 5 h. The resulted mixture was centrifuged at 8000 rpm for 25 min, the sedimented pale brown granules ZnO(NPs) was centrifuged filtered and dried at 80 °C for 7-8 h.

Characterization of synthesized green zinc oxide nanoparticles (gZnO NPs):

Zinc oxide nanoparticles were characterized using dynamic light scattering (DLS) assay to confirm particle size. Size assay was made in triplicate with a Malvern Zetasizer Nano ZS Instruments operating with a He–Ne laser at a wavelength of 327nm and a detection angle of 90°; all samples were analyzed for 60 s at 25°C. To confirm shape and size, each sample was diluted with deionized water and 50 µl of each suspension was placed on a formvar-coated copper grid for transmission electron microscopy (TEM).

Preparation of green zinc oxide nanoparticles solution:

Just before use, the stock solution was diluted in saline and ultrasonicated (SolidState/Ultrasonic FS-14; Fisher Scientific) for15 min to prevent aggregation. The concentration of ZnO in the suspension was 25 mg/ml.

II-Animals

Seventy male white albino rats weighing 200-250 grams were used. Rats were kept under observation for 2 weeks. Balanced ration and water were *ad. Libitum*. Inflammation was induced by intra -planter injection of 0.1 ml of formalin with concentration of 6% solution in normal saline (Domenjoz et al., 1995).

Experimental design

Rats were randomly divided into 7 groups (each group 10 rats). Treatment protocol is designed in Table (1).

Table (1): Illustrating the experimental groups and treatments protocol:

Group 1 (Gr1)	Control negative (n=10)
Group 2 (Gr2)	Control positive for inflammation that injected with formalin into the intra planter paw of right hind paw (100 µl) (n=10).
Group 3 (Gr3) (low Dexa dose)	Formalin treated as in Gr2 and given dexamethasone at a dose of 2mg/Kg b. wt./ day, (i.m) for 5 days (n=10).
Group 4 (Gr4) (high Dexa dose)	Formalin treated as in Gr2 and given dexamethasone at a dose of 5mg/Kg. b.wt./ day, (i.m) for 5 days (n=10).
Group 5 (Gr5) (gZnONP)	Formalin treated as in Gr2 and given g ZnO NPs at dose of 25mg/kg b. wt./day, (i.p.) for 5 days (n=10).
Group 6 (Gr 6) (gZnONP/low Dexa)	Formalin treated as in Gr2 and given g ZnO NPs at dose of 25mg/kg b. wt./day, (i.p.) and dexamethasone at a dose of 2mg/kg b. wt. (i.m) for 5 days (n=10).
Group 7 (Gr7) (gZnOP/high Dexa)	Formalin treated as in Gr2 and given g ZnO NPs at dose of 25mg/kg b. wt./day, (i.p.) and dexamethasone at a dose of 5mg/kg b. wt. (i.m) for 5 days (n=10).

Measurement of edema of the injected paw

The diameters of the hind paws were measured earlier for obtaining the base line value before the formalin injection and then, those of the formalin injected paw edema were also measured for evaluating the dorsal plantar foot thickness of the metatarsal level by a caliper at 30min, 1hr, 2hr, 3hr, 4hr, 24hr and 5 days in each after the injection. Both hind paws are measured simultaneously. The ability of anti-inflammatory agents to suppress paw inflammation was expressed as a percent inhibition of paw edema and calculated according to the following equation (Rajput, 2004): Relative Paw Edema=[v2−v1/v1] ×100

Where:

v_1 = The animal paw volume before formalin injection.

v_2 = The paw volume after drugs and formalin injection at different time points.

III-Sampling:

Blood sampling and analysis

On days; 1 and 7 post last administration, blood samples were collected from the dorsal pedal vein of each rat for immunological (serum and whole blood with anti-coagulant) and biochemical studies (serum). Serum from the blood sample was harvested after centrifugation at 3000 rpm for 10 minute and stored at–20^{o}C for biochemical analysis.

Biochemical analysis; including: Serum gamma glutamyl transferase (GGT) (Thomas, 1998), serum total protein (TP) (Young, 1995), serum Triglyceride (TG) (Royer and Ko,1969), serum total cholesterol (TC) (Richmond, 1973), serum High Density Lipoprotein cholesterol (HDL-C) (NCEPR, 1995), serum Low Density Lipoprotein cholesterol (LDL-C) (Friedewald et al., 1972), serum Very Low-Density Lipoprotein cholesterol (VLDL) (Norbert, 1995), serum Glucose (Trinder, 1969). Serum Sodium and Potassium concentration using flame photometer 410 C, Corning, Halstead, England. (ASTM International standard. Standard test methods for chemical analysis of hydraulic cement (ASTM C114-09) West Conshohocken, Pa, USA, 2009).

Immunological study; including measurement of lysozyme activity according to (Schultz, 1987), phagocytic activity of neutrophils for innate immune response assessment using dextran, 500,000 M.W from Sigma (Wilkinson, 1981).

Tissue sampling and examination

Tissue samples from spleen and injected paws were collected at days1 and 7 post last treatments for histopathological examination. Tissues were fixed in 10% buffered formalin and dehydrated in a graded series of alcohol, cleared in xylene, and embedded in paraffin wax. Multiple sections from each block were prepared at 5 μm and stained with hematoxylin and eosin (H&E) for histopathological studies (Survarna et al., 2012). Another set of spleen sections on positively charged coated slides were used for immune-histochemistry technique; using primary antibody of CD30. Technique was carried out according to

kit pamphlet' instructions. Bone marrows were harvested from femur of different groups and prepared freshly to estimate the status of DNA using Comet assay according to Singh et al., (1988).

1V- Statistical analysis

All data were expressed as means ± standard error (SE) and were subjected to statistical analyzed using one -way analysis of variance (ANOVA) according to Krystal (2004) Statistical significance was acceptable to a level of $P \leq 0.05$.

Results

I- Green synthesized Zinc Oxide Nano-particles examination

Transmission electron microscope (TEM) revealed the size and morphology of the synthesized particles. The complexes appear semi-cuboidal with a mean size that is inferior to 50 nm as can be seen in Fig. 1. This Figure undoubtedly indicate the morphology of the particles to be cuboidal. The size of the nanoparticle was determined to be ranged with 7-25nm diameter by transmission electron microscopy.

II- Edematous inflammatory reaction features

Callipered sizes of the injected paws in different groups at different time intervals were recorded in Table (2). The dexamethasone (2 and 5mg/Kg) significantly inhibited formalin-induced rat paw edema as compared to the inflamed group (Gr 2). Also, it was shown that there was significant difference between 5th, 6th and 7th groups and the inflamed group (Gr2) in inhibiting formalin-induced swelling (Table 2). There were significant differences in thickness of intra plantar into the right hind paw of rats between 3rd and 4th groups (2 and 5mg/Kg of dexamethasone) and the 5th, 6th and 7th groups in inhibiting formalin-induced swelling (Table 2). After 1-day post treatments ending the thickness of intra plantar into the right hind paw of rats were close to control group (Gr 1).

III - Biochemical parameters

The values of biochemical measurements of groups are shown in Tables (3 & 4). One day post treatment, inflamed group (Gr 2) showed a significant increase in plasma levels of,

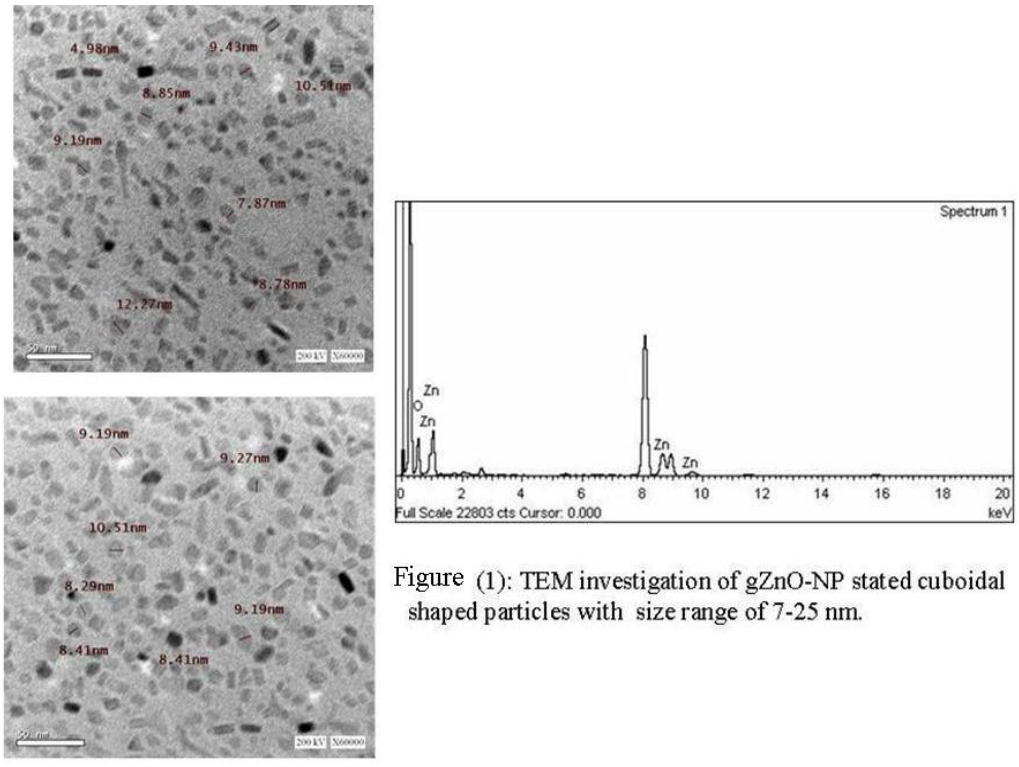

Figure (1): TEM investigation of gZnO-NP stated cuboidal shaped particles with size range of 7-25 nm.

gamma glutamyl transferase (GGT), glucose, triglyceride (TG), total cholesterol (TC), Low -Density (LDL), Very low-density (VLDL) with significant increase in sodium (Na), while significant decreased in High - Density (HDL), total protein(TP) and potassium (K) compared to control group (Gr1) ($p<0.05$) Tables (3 & 4).

When compared these parameters of Dexamethasone treated groups (Gr 3 and 4) with inflamed group (Gr 2), improve in all parameters (Tables; 3 & 4) could be noticed.

Dexamethasone at low dose (Gr3) revealed elevation of TG, TC, HDL, and glucose, but these differences failed to reach significance at the $P<0.05$ level, significant increase in GGT and TP when comparing the treated group (Gr3) with control group (Gr1), significant decline K Value in Gr3 when comparing with control group (Gr1). There was no significant change in LDL, VLDL and Na in Gr3 when comparing with control group (Gr1). Dexamethasone at high dose (Gr4) showed non –significant elevation of TG, TC, glucose and GGT levels, significant increase in HLD, TP and significant decrease in k level. There was no significant change in the level of LDL, VLDL and Na when comparing the treated group (Gr4) with control group (Gr1), Table (3 & 4).

In Gr 5, the Plasma levels of TC, TP and GGT were significantly increased, while the K levels were decreased, also mild increase in TG, HDL, VLDL and glucose and Na did not change when compared with Gr1. In Gr 6 and 7, biochemical parameters were slight change when compared with Gr1 (Tables; 3 & 4). These results indicated that activity of all parameters in Gr 5, 6 and 7 were improved compared with inflamed group (Gr2).

IV- Immunological parameters

Regarding to lysozyme activity, the results in Table (5) showed significant increase in lysozyme activity at 1day post treatment in Gr2, Gr3, Gr4, Gr5 while Gr6 and Gr7 showed non-significant increase when compared to control negative group Gr1. The daily administration of dexamethasone (2mg/kg and 5mg/kg) in groups Gr3 and Gr4 led to a statistically significant decrease in lysozyme activity compared to inflamed group Gr2. Green ZnO-NPS alone or with both doses of dexamethasone showed a significant decrease of lysozyme activity (Table; 5) when compared to groups 3 and 4 (dexamethasone alone). At 7 days post treatment, all groups showed significant decrease in lysozyme activity compared to control positive group Gr2, while Gr4 recorded significant decrease in lysozyme compared to control negative group Gr1. At the end of the experiment, inflamed group Gr2 showed significant decrease in phagocytic percentage and phagocytic index compared to control negative group Gr 1 while, Gr5, Gr6 and Gr7 showed significant increase in these parameters. Insignificant increases in phagocytosis of neutrophils parameters was recorded in Gr3 and Gr4 compared Gr2. Also, there was significant decrease in phagocytic percentage and in phagocytic index in Gr 4 compared to control negative group Gr1 (Table; 5).

Table (2): The callipered sizes of injected paws in different groups at chosen intervals along the experimental periods (n=10).

Groups	30min	1hr	2hr	3hr	4hr	24hr (from formalin injection)	1-day post treatments ending
Gr (1)	3.8±0.09	3.79±0.09	3.73±0.073	3.77 ±0.07	3.75±0.1	3.78±0.009	3.71±0.07
Gr (2)	5.12±0.12 (34.74%)	5.23±0.052 (37.99%)	5.28±0.09 (41.55 %)	5.13±0.098 (36.07 %)	5.11±0.09 (36.00%)	4.89±0.1 (29.37 %)	4.65±0.126 (25.34 %)
Gr (3)	4.88±0.097 (28.42%)	5.21±0.087 (37.47%)	4.98±0.06 (33.51 %)	4.87±0.04 (29.18 %)	4.79±0.08 (27.73%)	4.41±0.21 (16.67 %)	3.8±0.198 (2.43 %)
Gr (4)	4.59±0.29 (20.79%)	5.14±0.15 (35.62%)	4.94±0.07 (32.44 %)	4.88±0.08 (29.44 %)	4.5±0.21 (20.0 %)	4.46±0.22 (17.99 %)	3.9±0.077 (5.12 %)
Gr (5)	4.61± 14 (21.32%)	4.4±0.086 (16.09%)	4.32±0.17 (15.8 %)	4.26±0.16 (13.0 %)	4.24±0.16 (13.07%)	4.08±0.08 (7.94 %)	3.86±0.087 (4.04 %)
Gr (6)	4.66±.15 (22.63%)	4.5±0.067 (18.73%)	4.47±0.12 (18.64 %)	4.3±0.07 (18.56 %)	4.27±0.16 (13.87%)	4.24±0.17 (12.17 %)	3.76±0.17 (1.35 %)
Gr (7)	4.73±.11 (24.47%)	4.49±0.06 (18.47%)	4.41±0.09 (18.23 %)	4.32±0.07 (14.59 %)	4.24±0.16 (14.0 %)	4.21±0.17 (11.38 %)	3.99±0.65 (7.55 %)

Means in the same column bearing different letters superscripts differ significantly ($P<0.05$).

Table (3): The mean values of triglyceride and lipid profile of serum rats of different groups (n=10).

Groups	Trigly (mg/dl)		TC (mg/dl)		HDL-C (mg/dl)		LDL-C (mg/dl)		VLDL-C (mg/dl)	
	1day	7 days	1day	7 days	1day	7 days	1day	7 days	1day	7 days
Gr (1)	92.3 ±3.6^{a}	92.3 ±3.6^{ab}	86.68 ±3.47^{a}	86.68 ±3.47^{abc}	36.14 ±0.89^{a}	35.82 ±0.65^{a}	31.06 ±2.79^{a}	31.07 ±2.5^{a}	18.46 ±0.71^{a}	18.46 ±0 .71^{a}
Gr (2)	108.4 ±4.03^{b}	101.71 ±5.7^{b}	98.69 ±3.67^{b}	93.35 ±4.26^{b}	30.7 ±0.72^{b}	32.31 ±0.92^{b}	46.32 ±5.67^{b}	40.72 ±3.38^{b}	21.28 ±0.90^{b}	20.34 ±1.15^{a}
Gr (3)	99.05 ±2.98^{a}	93.77 ±3.0^{ab}	89.74 ±2.08^{a}	87.12 ±4.01^{abc}	38.14 ±1.55^{ac}	35.71 ±2.06^{ab}	31.55 ±2.8^{ac}	32.66 ±2.87^{a}	18.19 ±0.51^{ac}	18.75 ±0.6^{ab}
Gr (4)	98.46 ±2.51^{a}	92.84 ±2.22^{ab}	90.81 ±1.97^{a}	85.8 ±4.39^{abc}	39.87 ±1.21^{c}	36.41 ±1.52^{ab}	32.0 ±1.85^{ab}	30.17 ±1.69^{a}	19.03 ±0.73^{ab}	18.57 ±0.4^{ab}
Gr (5)	94.29 ±3.22^{a}	91.34 ±3.96^{a}	100.0 ±3.31^{b}	94.46 ±4.07^{ab}	37.99 ±0.9^{a}	34.72 ±1.44^{ab}	32.09 ±1.31^{a}	32.50 ±1.22^{a}	19.51 ±0.19^{ac}	18.27 ±0.79^{b}
Gr (6)	95.01 ±2.77^{a}	93.46 ±2.72^{ab}	89.96 ±3.09^{a}	85.08 ±2.42^{abc}	35.76 ±1.33^{ad}	35.52 ±0.84^{ab}	33.82 ±3.48^{ac}	31.29 ±1.41^{a}	18.86 ±0.64^{ac}	19.89 ±0.54^{ab}
Gr (7)	94.23 ±1.29^{a}	92.17 ±0.75^{ab}	88.79 ±3.21^{a}	81.62 ±2.96c	36.22 ±1.98^{ad}	35.95 ±0.44^{ac}	33.77 ±2.15^{ac}	30.14 ±1.01^{a}	18.81 ±0.55^{ac}	18.6 ±0.53^{ab}

Means in the same column bearing different letters superscripts differ significantly (P<0.05). Trigly=Triglyceride (mg/dl). TC=Total cholesterol. HDL-C= High density lipoprotein (mg/dl). LDL-C= low density lipoprotein (mg/dl). VLDL_C = very low-density lipoprotein (mg/dl). All recorded values were within the permissible limits of rats according to AOAC (2015).

Table (4): The mean values of some biochemical parameter and mineral of serum rats of different groups (n=10).

Groups	Trigly (mg/dl)		TC (mg/dl)		HDL-C (mg/dl)		LDL-C (mg/dl)		VLDL-C (mg/dl)	
	1day	7 days	1day	7 days	1day	7 days	1day	7 days	1day	7 days
Gr (1)	92.3 ±3.6^{a}	92.3 ±3.6^{ab}	86.68 ±3.47^{a}	86.68 ±3.47^{abc}	36.14 ±0.89^{a}	35.82 ±0.65^{a}	31.06 ±2.79^{a}	31.07 ±2.5^{a}	18.46 ±0.71^{a}	18.46 ±0.71^{a}
Gr (2)	108.4 ±4.03^{b}	101.1 ±5.7^{b}	98.69 ±3.67^{b}	93.35 ±4.26^{b}	30.7 ±0.72^{b}	32.31 ±0.92^{b}	46.32 ±5.67^{b}	40.72 ±3.38^{b}	21.28 ±0.90^{b}	20.34 ±1.15^{a}
Gr (3)	99.05 ±2.98^{a}	93.77 ±3.0^{ab}	89.74 ±2.08^{a}	87.12 ±4.01^{abc}	38.14 ±1.55^{ac}	35.71 ±2.06^{ab}	31.55 ±2.8^{ac}	32.66 ±2.87^{a}	18.19 ±0.51^{ac}	18.75 ±0.6^{ab}
Gr (4)	98.46 ±2.51^{a}	92.84 ±2.22^{ab}	90.81 ±1.97^{a}	85.8 ±4.39^{abc}	39.87 ±1.21^{c}	36.41 ±1.52^{ab}	32.0 ±1.85^{ac}	30.17 ±1.69^{a}	19.03 ±0.73^{ac}	18.57 ±0.4^{ab}
Gr (5)	94.29 ±3.22^{a}	91.34 ±3.96^{a}	100.06 ± 3.31^{b}	94.46 ±4.07^{ab}	37.99 ±0.9^{a}	34.72 ±1.44^{ab}	32.09 ±1.31^{a}	32.50 ±1.22^{a}	19.51 ±0.19^{ac}	18.27 ±0.79^{b}
Gr (6)	95.01 ±2.77^{a}	93.46 ±2.72^{ab}	89.96 ±3.09^{a}	85.08 ±2.42^{abc}	35.76 ±1.33^{ad}	35.52 ±0.84^{ab}	33.82 ±3.48^{ac}	31.29 ±1.41^{a}	18.86 ±0.64^{ac}	19.89 ±0.54^{ab}
Gr (7)	94.23 ±1.29^{a}	92.17 ±0.75^{ab}	88.79 ±3.21^{a}	81.62 ±2.96^{c}	36.22 ±1.98^{ad}	35.95 ±0.44^{ac}	33.77 ±2.15^{ac}	30.14 ±1.01^{a}	18.81 ±0.55^{ac}	18.6 ±0.53^{ab}

Means in the same column bearing different letters superscripts differ significantly (P<0.05). Glu= Glucose (mg/dl). Tp = total protein (g/dl). GGT = gamma-glutamyl transferase (U/L). Na= sodium (mmol/L). K= potassium (mmol/L). All recorded values were within the permissible limits of rats according to AOAC (2015).

Table (5): Serum lysozyme activity, phagocyte % and phagocyte index of neutrophil in experimental groups (n=10).

Groups	Lysozyme(µg/ml)		At the end of experiment	
	1day post treatment	7days post treatment	Phagocytic %	Phagocytic index
Gr (1)	381 ±14.2^{a}	432±9.1^{a}	52±1.30^{a}	1.5±0.06^{a}
Gr (2)	581 ± 15.12^{b}	521±6.2^{b}	46. 2±1.49^{b}	1.12±0.06^{b}
Gr (3)	480 ± 13.7^{cd}	440±6.08^{a}	49.5±1.5^{ab}	1.22±0.05^{b}
Gr (4)	461± 12.7^{d}	335±6.5^{c}	47±1.1^{b}	1.32±0.02^{c}
Gr (5)	450±12.8^{c}	401±7.35^{acd}	61±1.54^{c}	1.6±0.04^{ad}
Gr (6)	393 ± 12.11a	420±6.86 acd	56.11±1.8 cd	1.68±0.02 d

Gr (7)	405 ±15.4 a	431±6.52 a c	65±1.2 d	1.59±0.06 ad

Means in the same column bearing different letters superscripts differ significantly (P<0.05)

V-Histopathological and immunohistochemical study (Fig. 2-5 and Table 6)

Spleens: Spleen examination revealed demarcations of lymphoid follicles into germinal centers and mantel zones with proliferation and separation of selecting B- lymphocytes into the mantel zones at variable degrees in different groups. At 1-day interval; collection of B-lymphocytes with development of germinal centers were clear in Gr 2 & 5 (Fig.2, A & D); while moderate development of germinal centers was detected in Gr 3 & 6 (Fig.2, B & E) and ell development in Gr 4 & 7 (Fig.2, C & F). Mantle zones development; that represent proliferating B-lymphocytes were wide in Gr 3 & 5 (Fig.2, B& D), while moderate sized zones were detected in Gr 4 & 6 (Fig.2, C & E) and narrow in Gr 2 & 7 (Fig.2, A & F). CD30 as a marker of activated T-lymphocytes (in the inter-follicular zones) that will further differentiate into Th2; exhibited significant degree of reactivity in the different groups. Severe degree of reactivity was clear in Gr 2 (Fig.3, A) & intense in Gr 3 (Fig.3, B). Moderate reactivity was detected in Gr 5 (Fig.3, D) with marked reduction in Gr 4 & 6 (Fig.3, C & E), while there was complete absence of reactivity in Gr7 (Fig.3, F). At 7 days intervals post treatment; the histological features referred to return to normal criteria comparable with control negative group.

Skin: At 1-day interval, dermal macrophages showed variable degree of infiltrations with a common feature of brown–black granules incorporation Similar reactivity was recorded in Gr 4 & 5 (Fig. 4, C & D) (moderate) followed by Gr 2 & 3 (Fig. 4, A & B) (marked) and Gr 6 & 7 (Fig.4, E & F) (less). At 7 days intervals: all groups showed almost the same intense degree of dermal macrophages infiltrations with marked diminished character.

Bone marrow: At 1 day, no significant changes could be detected in Gr: 2 & 3 (Fig.5, A & B). Gradual increase in the degree of DNA damage was recorded in up sequence in the following order Gr: 4, 5 & 6 (Fig.5, C, D & E), while group 7 (Fig.5, F) showed genotoxic effect in form of micro-nucleus development; mainly in neutrophils. At 7 days interval the previously detected genotoxic effect was markedly reduced in all investigated groups.

Discussion

In the biological method, plant extracts are used for controlled and precise synthesis of several metallic nanoparticles (Rajiv et al., 2013). The green synthesis of metal nanoparticles used biological material as ecofriendly, non-toxic and safe biosynthetic process (Caruthers et al., 2007, Salam et al., 2012, Moritz et al., 2013, Nath and Banerjee, 2013). In the first part of the study, the synthesis of ZnO NPs using the olive plant; so far there is no report on the synthesis of nanoparticles using olive leaf extracts. In this paper, we report the synthesis of zinc oxide nanoparticles using water olive leaf extracts as a simple, low cost and reproducible method (Salam et al., 2012).

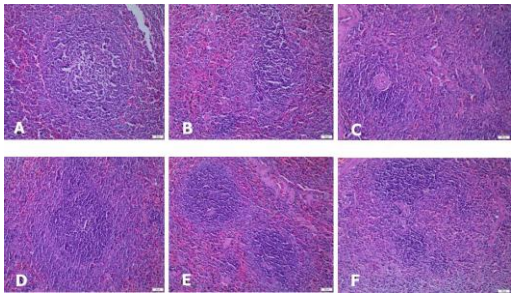

Fig. 2. The development of white pulp criteria as an indicator for B- cell response, at 1-day interval. Well-developed germinal centers and narrow mantle zones (A/ Gr2), moderate developed germinal centers and wide mantle zones (B/ Gr3), less developed germinal centers and moderate sizes mantle zones (C/ Gr 4), well developed germinal centers and. wide mantle zones. (D/ Gr5), moderate diameters germinal centers and moderate sized mantle zones (E/ Gr6) and ell degree of development of germinal centers and associated with narrow mantle zones (F/ Gr7). H&E, X 200.

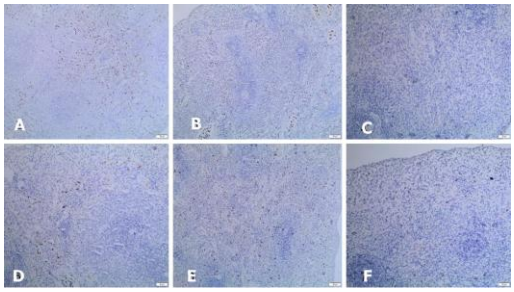

Fig. 3: The reactivity in different groups was restricted to intra follicular T-cell zone), at 1-day interval. Severe (A/ Gr 2), intense (B/ Gr3), marked reduction in the reactivity (C/ Gr4), moderate (D/ Gr5), reduction in the reactivity (E/ Gr6) and complete absence of the reactivity (F/ Gr7). IHC "CD33"- X 200

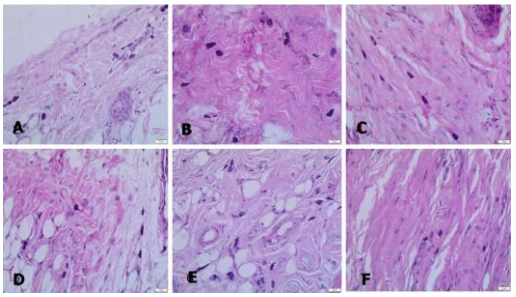

Fig. 4: Dermal macrophages showed variable degree of infiltrations at 1-day interval. Similar reactivity was recorded in C & D/ Gr 4 & 5 (moderate), followed by A & B/ Gr 2 & 3(marked) then E & F/ Gr 6 & 7 (severe). H&E X 400

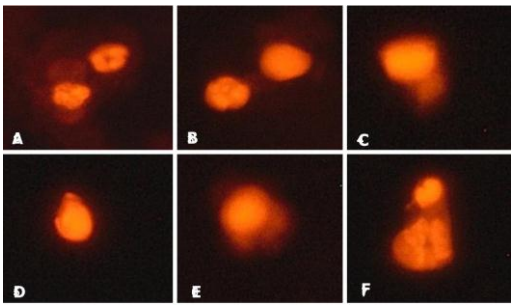

Fig. 5: Comet assay at 1-day interval revealing no significant changes could be detected in A/ Gr2 & B/Gr3.Gradual increase in the degree of DNA damage was recorded in up sequence in the following C/ Gr: 4, D/ Gr 5 & E/ Gr 6, while F/ Gr 7 showed genotoxic effect in form of micro nucleus development.

TEM image confirms the formation of zinc oxide nanoparticles and it has an average size about 15 ± 10 nm, which is very similar to previous studies (Borchert et al., 2005, Jones et al., 2008 and Bar et al., 2009). Inflammation is a natural complex protective biological response by the living organism to remove harmful stimuli such as pathogens, damaged cells or irritant and to initiate the healing process for tissues (Ferrero et al., 2007). Edema formation, leukocyte infiltration and granuloma formation represent such components of inflammation (Mitchell and Cotron, 2010). Formalin induces paw edema is closely resembles human arthritis (Banerjee et al., 2000). The inflammatory effect of formalin is biphasic; where first phase is the direct effect of formalin, which involves neurogenic pain. The second phase is involved in the inflammatory reactions mediated by prostaglandin, serotonin, histamine, bradykinin and cytokines, such as interleukin-1 beta, interleukin-6 tumor necrosis factor-alpha eicosanoids, and Nitric Oxide (Fu et al., 2001). Edema formation in the paw is the result of a synergism between various inflammatory mediators that increases vascular permeability and blood flow (Lalenti et al., 1995). Our results of the formalin -induced hind paw edema test were summarized in Table (2). Variable dexamethasone dosage reduced the edema after the two and three h from formalin injection, by 33.51, 29.18% (Gr3) and 32.44, 29.44% (Gr4). High dose of dexamethasone injection was more effective to inhibit inflammatory reaction. Our results agreed with Magdalena et al., (2015). Dexamethasone is a potent anti-inflammatory drug capable of inhibiting edema formation. Al-Asmari and Abdo (2006) found that dexamethasone (1mg/kg) was known to indirectly inhibit phospholipase A2 (PLA2) action. Furthermore; it directly acted on leucocytes and other cell types inhibiting the release of cytokines and other inflammatory mediators (Angeli et al., 1999). Similar actions were observed in the kinetics and cell composition of the inflammatory infiltrate in rat paw. Dias Araújo et al., (2007) referred to the effectiveness of dexamethasone (1mg/kg) in reducing the paw edema; dexamethasone can inhibit not only the production of lipid mediators, but also the rolling and adhesion of leukocytes to endothelial cell surface induced by TNF-a (Zhang and Thorlacius, 2000). In this research, the administration of ZnO NPs only (Gr5) reduced the edema after 1st h formalin injection by 16.09%. This result was explained with Jinquan et al (2017) who found that ZnO-NPs treatments show significant dose-dependent efficacy for reduction of oxidative stress in inflamed mice and suppress proinflammatory cytokines (IL-1β and TNF-α). We found that administration of ZnO NPs and dexamethasone (2 and 5mg/kg) caused a reduction the edema after 1st and 2 nd hr after formalin injection by 18.73,18.47%, 19.84 and 18.23% in comparison to the inflamed group (Gr2) respectively. Mechanism of ZnO-NPs as anti- inflammatory may attributed to their ability to induce the generation of reactive nitrogen species and overexpression of Cox-2, iNOS, pro-inflammatory cytokines (IL-6, IFN-$_{\gamma}$, TNF-ά, IL-17 and regulatory cytokine IL-10) and MAPKS which were found to be inhibited after blocking

internalization of ZnO-NPs through caveolae receptor pathway (Roy et al., 2014). During our work, we noticed that the inflammatory edematous reaction after formalin injection was reduced in groups Gr3 and Gr4 (Dexa -alone) while greater response was recorded in Gr6 and Gr7(Dexa with ZnoNPs). In this study, when compared the parameters of inflamed group (Gr2) with control negative group (Gr 1) significant change in all parameters could be observed. Our results were in agreement with reports from other researchers, Agnel and Shobana (2012) who found that, administration of formalin (0.1ml/kg bw) in rats resulted in a significant increase in the levels of lipid peroxide, hydroxyl proline, hexosamine, serum enzymes, blood glucose level and decrease total protein, also increase in the paw thickness. Agnel and Shobana (2012) recorded decrease in serum protein level after formalin and explain this result, proteins are the building block of amino acids. The propagation of free radical from inflamed tissue can bring many adverse reactions leading to extensive tissue damage. Lipids, proteins, DNA are very susceptible to attack by free radicals. The level of serum protein content is lowered in arthritis. The proteins were clearly changed the perception of the pathogenesis of inflammation which has been reported earlier (Weissman, 1967). Chronic inflammation is known to stimulate protein metabolism in animals. Micheline et al., (1996) reported that, formaldehyde injection resulted in an inhibition of ursodeoxycholate-induced bicarbonate secretion in rats and in a marked increase in glucose. Bono et al., (2010) and Szende and Tyihák (2010), suggest that formalin can induce oxidative stress by increasing the formation of reactive oxygen species (ROS). The increase in serum glucose levels in fish exposed to formalin was noted (Yildiz et al., 2009). Formaldehyde is a very reactive one-carbon compound, can react with lipids, proteins, and nucleic acids which are cellular components. Afrin et al., (2016) reported significantly increased in AST and ALT level after exposure to formaldehyde, due to impairment of hepatic functions. The present study indicates to improvement in all parameters of dexamethasone treated groups (Gr 3 and 4) as compared with inflamed group (Gr 2). Dexamethasone enhances hepatocytes viability and improves the expression of liver-specific genes in liver diseases, it has been used as an anti-inflammatory drug in the treatment of hepatitis and cirrhosis through suppression of collagen synthesis and gene expression by fibroblasts (Ki et al., 2005). Thus, the beneficial effect of dexamethasone in ascites formation may be attributed to its anti-inflammatory effect and to a decrease in the production of vasoactive substances from inflammatory cells, but not to improve liver functions (Melgert et al., 2000). Our results revealed significant decrease of GGT in Gr3 and Gr4 when compared Gr2, that agreed with Halil et al., (2006), who also reported significant decrease in ALP, AST and ALT, and increase in levels of these enzyme when compared with control group (-ve). In present study, all biochemical parameters in Gr3 and Gr4 were decreased but still not reach to control level in Gr1. Many literatures recorded that dexamethasone injection causes elevated levels of TG, TC, HDL,

glucose and GGT and these changes are receivable in acute cases (Kaukonen et al., 2014 and Mizen et al., 2017). Acute effects of 3 mg dexamethasone (twice daily simulating acute stress) in young men induced lower highly sensitive C-reactive protein levels and increased HDL; while LDL, NEFA and TG were not altered. Glucocorticoids reduce hepatic lipase and CETP and increased ApoAi resulting in elevated HDL cholesterol (Ross and Marais, 2014). Pervious study is partially agreeing our result. Mizen et al., (2017) reported increase in glucose concentration after receiving 0.05 mg/kg of dexamethasone (i.m.) to healthy horses; this increasing of glucose may be related to the effect of corticosteroids on carbohydrate metabolism (McMahon et al., 1988). One potential explanation for dexamethasone-induced type B hyperlactatemia is the hyperglycemic effect of glucocorticosteroids. Glucocorticoids are stress hormones that are essential to mammalian glucose homeostasis (Kuo et al., 2015). By stimulating hepatic gluconeogenesis and inhibiting glucose uptake and utilization in muscle and adipose tissue, they allow plasma glucose concentrations to increase, an adaptation that might be important in maintaining optimal brain function in times of stress (Kuo et al., 2015). Our result demonstrated significant decrease in k level while no change in sodium level, that in agreement with Yahi et al., (2017) and Amar et al., (2013), who found that Dexamethasone (low and high dose) causing decrease in potassium, this result could be attributed to the nature corticosteroids that have weak mineralo- corticoid properties, so increasing renal tubular sodium re-absorption and increasing potassium excretion. The mechanism underlying the decrease in K + is not clear; however, the decrease could be due to increased urinary K + excretion without corresponding effects on sodium or calcium excretion. A similar observation was reported by Johnson et al., (2009) in rats where dexamethasone injection produced 70% increase in urinary K + excretion. One of the important indicators for assessing of zinc oxide nanoparticle as anti-inflammatory effect status is decrease signs of inflammation as edema and biochemical parameters. In the present study, the administration of ZnO NPs (group Gr5) cause significantly ($P<0.05$) improvement on serum parameters in comparison to control positive group (Gr2). This results accordance the findings of Bashandy et al., (2018), they Suggested that ZnO NPs (5, 7.5, and 10 mg/kg, i.p) lowered tissue malondialdehyde, plasma inflammatory markers (TNF-á, IL-6), liver enzymes (gamma-glutamyltransferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and kidney function parameters. In the present study, the decreased GGT activity levels in liver indicated that the presence of oxidative stress and lipid peroxidation response were generated by ZnO NPs exposure. It has been reported that oxidative stress mediated DNA damage and cytotoxicity induced by Zn-NP in HepG2 cells (Sharma et al., 2011 and Sha et al., 2014). Mechanism of the generation of the oxidative stress after NPs treatment is not clear, but Singh et al., (2007) indicated that it is related to the large

particle surface area. During our work, the glucose level was reduced, and this data is adopted with Weidong et al., (2018) who said that ZnO NPs caused glucose reduction which indicated that aerobic metabolism and the tricarboxylic acid (TCA) cycle were perturbed in the liver. In this study, comparison the parameters of group Gr5 with control group (Gr 1) revealed changes in some parameters; TC, HLD, TP, GGT and K. The findings of the current study in partial agreement with Fazilati (2013) who reported that zinc oxide nanoparticles (25-200 mg) had significantly increased ($P<0.05$) activity of enzymes in male rats. The increase in the enzymes activity may be attributed to increased concentrations of cholesterol by nano-ZnO (Guyton and Hall, 2006). Meanwhile, the rats that received ZnO NPs showed a significant elevation in the serum TC and HDL when compared with control negative group. It is also congruent with the findings of (Hazim et al., 2011) who showed that supplementation with ZnO NPs in broilers increased plasma total cholesterol and HDL. The change in the cholesterol levels of blood may be due to the incorporation of zinc in the integral part of some metalloenzymes that are severed in lipid digestion and absorption. Olechnowicz et al., (2018) reported that zinc supplementation results in the total cholesterol, LDL cholesterol, and triglycerides decreasing, and the HDL cholesterol increasing in patients, via increases hepatocyte activity and improves lipid metabolism in the liver. In the present study, glucose levels were significantly reduced after ZnO treatment (Gr5), while treatment with both doses of dexamethasone increased glucose content when compared with ZnO treatment (Gr5). This was attributed to the hyperglycerism of dexamethasone. Also, zinc inhibits of glucagon secretion in response to high glucose concentrations. Glucagon is a hormone secreted by a-cells of the pancreas, which increase the glucose blood level during hypoglycemia. When the glucose concentration decreases, zinc is released from the b-cells with insulin, triggering glucagon excretion which regulates glycogen breakdown and gluconeogenesis and decreases at the same time triglyceride synthesis by the liver (Slucca et al., 2010 and Slepchensko and James 2013). Lysozymes are proteins of low molecular weight found in polymorph nuclear cells and synthesized also in mononuclear cells. Lysozymes are considered as a member of innate humoral factors that elaborate from body and show increase in their concentration (Weir, 1983). It is a cornerstone of innate immunity, found in abundance in the blood and liver, and in body secretions, at mucosal surfaces in addition to professional phagocytes including; macrophages, neutrophils, and dendritic cells (Callewaert and Michiels, 2010). In respect to lysozymes, relatively little is published in the recent literature on the impact of glucocorticoids on the expression of lysozyme. In this study all, experimental groups except control negative group (Gr1) revealed significant increase in lysozyme activity at 1-day post treatment but at 7 days high dose dexamethasone treated group (Gr4) exhibited significant decrease in lysozyme. These results partially agree with Panarelli et al., (1994) who recorded glucocorticoids

inhibit translation of the lysozyme gene. In the present study dexamethasone, at high dose (Gr4) at 7th day, inhibited lysozyme synthesis to a similar degree for all types of white blood cells. A possible mechanism that might explain our findings is proposed. It is known that increased levels of glucocorticoids inhibit virtually all functions of macrophages and monocytes (Dupont et al., 1985, Reinhart and Ackerman, 1983). Macrophages and neutrophils are the principal sources of lysozyme; high dose of dexamethasone can inhibit the production and secretion of lysozyme resulting in decreased levels of the enzyme (Elsbach and Weiss, 1992). Increased lysozyme at 1day post inflammation in all groups (except control negative group) might be attributed to inflammation induced by formalin injection. Santos et al., (2004) reported biphasic leucocytic response following formalin injection into peritoneal cavity; the first peak from 2-4 hours was characterized by mixed polymorph nuclear and lymphocytic cell population representing 100-220% and 55-60% respectively whereas the second peak was characterized by marked increase in lymphocytes at 24 hours representing an increase of 230%. Mishra et al., (2000) and Bargagli et al., (2008) stated that increase in serum lysozyme associated with certain chronic inflammatory conditions. Although lysozyme is important for driving a pro-inflammatory response, lysozyme also plays a role in limiting inflammation systemically, resulting in decreased inflammatory driven Ganz et al., (2003). Regarding to neutrophils; that are the primary line of defense in innate immune response. The data cleared in Table (5) indicated to the significant decrease in phagocytic percent and phagocytic index in high dose dexamethasone treated group (Gr4) compared to control negative group. This agrees with Alabdullah et al., (2015) who found that (in vitro studies) consistently decreased neutrophil function as indicated by decreased percentage neutrophils phagocytic E-coli, decreased number of E-coli phagocytized by neutrophil and decreased neutrophil O2(-) production in bovine neutrophils treated with dexamethasone, compared to controls. Moreover, Roth and Kaeberle (1981) reported dexamethasone administration (a single large dose) to cattle induced depress the ability of neutrophils to ingest staphylococcus aureus. Dexamethasone has also been reported to impair the oxidative metabolism of bovine neutrophil. Also, Cooper et al., (1972) found that corticosteroids inhibit ingestion by human neutrophils. The depression of oxidative metabolism indicates that an important component of the neutrophils bactericidal mechanism is impaired by dexamethasone administration (Fuenfer et al., 1979). Similarly, Chen et al., (2017) found that treatment with dexamethasone in mice induce negative effects on the numbers of both natural killer cell and T- lymphocytes in dose – dependent manner. Ardiana and Rifa (2015) found that dexamethasone (at dose of 0.5mg/kg B.W. i.p.) did not significantly decrease B-lymphocytes but the dose of 10mg/kg B.W did. On the other hand, glucocorticoids enhance innate immunity while suppressing adaptive immunity; these drugs enhance the survival and /or function of neutrophils and alveolar macrophages but induce the apoptosis of airway

dendritic cells (Schleimer, 2004). Dexamethasone administration in man has reported to enhance neutrophil-mediated antibody-dependent cell - mediated cytotoxicity (Parrillo and Fauci, 1978). Other studies reported that glucocorticoids treatment did not cause a global suppression of monocytic effect or function but results in differentiation of a specific anti-inflammatory involved in resolution of inflammatory reactions (Ehrchen et al., 2007). In our results Gr5 administered g ZnO NPs alone and /or with dexamethasone at both doses (Gr6 and Gr7) showed significant increase in phagocytic % and phagocytic index compared to control negative group. These results indicate to the stimulatory effects of g ZnO NPs on immune system, similar results obtained by Roy et al., (2014) who found that intraperitoneally administered ZnO NPs with ovalbumin induce elevation of ovalbumin specific immunoglobulin (IgG1) and (IgGE). Sahoo et al., (2014) reported that feeding broiler chicken with feed containing 0.06ppm Zn - NP produced higher antibody titer to SRBC when compared to control negative group. Unlike Kim et al., (2014) demonstrated that ZnO NPs (750 mg/kg/day, for 2weeks) induced suppression natural killer cell activity but did not alter the cell-mediated immune response, this finding not in accordance with the present study. Waston et al., (2015) recorded that intravenous administration of ZnO NPs induced transiently inhibition of kuffer cell phagosomal motility but did not alter bacterial clearance from the blood in rat model. Our findings of the innate immunity differ with the other results which may be due to the difference in the criteria of investigated ZnO NPs. Kononenko et al., (2015) stated that more attention should also be given to determining the mechanisms of interaction between nanoparticles and different components of the immune system to understand why the same nanoparticles stimulate certain immune functions and suppress other. Depending on the previous study, which stated that spleen is one of the main organs for ZnO NPs distribution (Choi et al., 2015) due to the non-specific uptake of metallic nanoparticles by reticuloendothelial cells (Lin et. al., 2015); so, we study, in a comparative manner, the potential effect of the investigating agents on; spleen lymphocytes components (systemic immune response of both humeral and cellular arms) and skin macrophages (localized immune response with mainly cellular immune response); in addition to their effect on bone marrow which represent the pool of immune cells production. The susceptive immune effect of ZnO NPs depend on the biological effect of Zinc as a trace element incorporated as a cofactor in more than 300 enzymes (Wellinghausen and Rink, 1998), in addition to the tendency ZnO NPs to incorporate with highly proliferated cells such as lymphocytes (Christa et al., 2015). In this study under the pathological section we try to elucidate the modulating effect of investigated therapeutic agents on spleenic components of both B and T-lymphocytes and their cross reactivity. The immune modulating effect on spleen lymphocytic components was clear in the different investigated groups (at variable degrees), which appeared in form of activation of T-

lymphocytes (periarteriolar sheath/PASL) followed by proliferation of follicular B lymphocytes with well demarcation of the germinal centers and development of the mantle zone. Development of lymphoid follicles could be attributed to the stimulation of mature B- lymphocytes under the effect of stimulated T- lymphocytes/PASL (T-B lymphocytes cross talk). Our study indicated to the stimulating effect of formaldehyde that was sustained up to 1week. ZnO NPs could not suppress the formaldehyde stimulating effect on B-lymphocytes, while dexamethasone therapeutic regime in the other groups had noticeable suppressive effect, which represented by variability of germinal centers development and further demarcation of mantle zones. Regarding to the immune reacitivity with CD30 (as a marker of activated T-lymphocytes- mediating NF-κB pathway) and its role in regulating the immune response; our study demonstrated down-regulation in the immunological stimulation of T-lymphocytes- mediate NF-κB pathway; through chemokine receptors exhibited by CD30 molecules. Estimation of immune reactivity of CD3 revealed that at the first day in g ZnO NPs group alone and / or associated with dexamethasone, while dexamethasone (alone) at 2 dose levels had less immune-suppressive effect. T-lymphocytes (CD30+/ Th2) are member of the tumor necrosis factor receptor mediate NF-kB and mitogen-activated protein kinase signals and physiologically expressed on a subpopulation of T helper cells (Th2) (Watanabe et al., 2011). Findings of van der Weyden et al., (2017) stated the role of CD30 in immune surveillance and cross-talk between B and T lymphocytes. Its expression has been proposed as a marker for Th2 phenotype and is correlated with allergen severity (Leonard et al., 1997); that in accordance with our results. T h2 lymphocytes are associated with humeral immunity and production of IL-4 and IL-5 (Jones et al., 1999). There was a reverse correlation between the germinal centers development and the degree of CD30 reactivity which also recorded by Gaspal et al., (2005) and explained to be a cause of loss of a sustained germinal center response with subsequent impairment in the secondary Abs production in mouse deficient in CD30; and hence the severity of affection is correlated with the expression of CD4+ / CD30+ (Leonard et al 1997). Hanley et al., (2009) had stated the immune modulating (activating) effect of ZnO NPs mainly on Th1 (T- cytotoxic) lymphocytes through innate and cell – mediated immunity. The ability of ZnO NPs to initiate Th1 differentiation is mediated by its ability to induce TNF-α (Lappin and Campbell, 2000). Our results clarify the immune – suppressive effect of g ZnO NPs on Th2 lymphocytes. The ability of ZnO NPs to induce proinflammatory cytokine expression is consistent with the recognized relationship between oxidative stress and inflammation, which is partially mediated by induction of the NF-κB transcription factor (Federico et al., 2007), with subsequent secretion of inflammatory cytokines such as IL-6 (Simón-Vázquez et al., 2016). Studying the effect of ZnO NPs on dendritic cells revealed activation and maturation with subsequent release of IL-6 and TNF-α upon exposure to

ZnO NPs (Heng et al., 2011 and Sahu, et al., 2014). Kim et al., (2014) reported the immunosuppressive effect of ZnO NPs as a sequence of innate immunity (natural killer cells) and consistently Th2 cytokines in ZnO NPs -fed mice. This immune suppressive effect could also be attributed to enhancements of phagocytic activity of macrophages and cytokines secretions by polymorphonuclear leukocytes (Wang, et al., 2014) and parallel with our findings. The present study refers to the impaired immune suppressive effect of dexamethasone on Th2-lymphocytes in a dose dependent manner; while g ZnO NPs had marked immune- suppressive effect which is enhanced when admixed with dexamethasone in correlation with the dose. The reverse relation between dexamethasone dose and T/ +CD 30 reactivity, could be attributed to the apoptotic effect of dexamethasone on T-lymphocytes as previously detected by Workman et al., (2012) and Xing et al., (2015). Regarding to dermal macrophage; the present study detected marked suppressive effect in g ZnO NPs group; Kim et al., (2014) demonstrated suppressed innate immunity of Zn on the natural killer cell activity. Similar suppressive effect was noticed in dexamethasone groups, while lost in admixed groups with g ZnO NPs, which give suspension of competitive pathway. The noticeable activation of dermal macrophage could be attributed to the modulation effect of dexamethasone on macrophage polarization and shifting effect from M1 (proinflammatory) to M2 (anti-inflammatory) subtypes (Fengm et al., 2017). With concern to the combination treatment of ZnO NPs with dexamethasone; dexamethasone a well-known cell-mediated immune suppressive cortical agent through inhibition of naive T-cell proliferation and IFN-α production with cytoplasmic-nuclear shuttling of NF-κB (Montesinos et al., 2012), this effect extends on dendritic cells and indirectly impair the Th1 development and interfere with the Th1-Th2 balance through IL-12 and/or IL-10 secretion by dendritic cells (He et al., 2010). Regarding to the effect of g ZnO NPs on the bone marrow, we could detect significant genotoxic effect using comet assay, Kumar et al., (2015) attributed this marked effect to the accessibility of ZnO NPs to the nuclei, and hence interaction with enzymes involved in detection of DNA breaks. Similar findings were also detected by Senapati et al., (2015) and attributed it to the induction of nitrosative effect on monocytes. While Sliwinska et al., (2015) indicated to oxidative DNA damage with strand breaks. Simón-Vázquez et al., (2016) attributed that to the high activity of ZnO NPs and the release of Zn2+ ions as the main mechanism of toxicity. Further illustration was provided by Syama et. al. (2014); and referred to the dissolution of ZnO NPs in the acidic lysosomal compartment with high level of ROS that lead to lysosomal membrane damage and activation of apoptosis cascade through caspase-3 &-7. Makumire et al., (2014) suggested that ZnO NPs are nascent particles and interact with DNA and protein, lead to production of complex proteins those resulted in cytotoxic harmful effect. In general, the small size of nano-particles helps them to evade the macrophage response (Roy et al., 2011). The cytotoxic effect of ZnO NPs on immune

cells could be attributed to their high proliferative activity with subsequent high level of ROS, which had marked determinant effect under the impairment effect of ZnO NPs on the anti-oxidant process (Hanley et al., 2008), so ZnO NPs induce toxicity in a cell- specific and proliferation-dependent manner with rapidly dividing cells being the most sensitive and quiescent cells being the least sensitive (Christa et al., 2015). Sliwinska et al., (2015) demonstrated this cytotoxic and even genotoxic effect mainly on lymphocytes. Another specification for cytotoxicity was given by Hanley et al., (2009) who stated that lymphocytes are the most resistant and monocytes are the most susceptible to ZnO-NP toxicity; and this toxicity depend on the interaction of ZnO NPs with cell membrane and the extent of free radical's formation. This genotoxic effect be more detectable and even lead to micro-nucleus induction in the treatment mixed groups (Gr6&7) which could be attributed to combination of apoptotic effect of dexamethasone (Workman et al., (2012) and Xing et al., (2015). and the DNA-strands breaking effect of ZnO NPs. At 7 days interval; there was no noticeable effect of the investigating agents (at different doses), that in accordance with Choi et al., (2015) who recorded the significant reduction in ZnO NPs distribution by 7th day post administration and hence a reverse genotoxic effect (Hackenberg et al., 2011) even at 24 hr post exposure.

Conclusion

In the present, when compared these parameters of Dexamethasone with ZnO NPs treated groups (Gr 6 and 7) with inflamed group (Gr 2) observed improvement in all parameters. The presented data indicates that Dexamethasone with ZnO NPs have synergistic significant anti-inflammatory activity. Due to the recorded genotoxic effect, further studies are recommended to confirm this synergistic effect as no literatures are recorded. At 7 days post last treatment, all groups showed improve biochemical parameter in all groups which indicate to the transient effect of the investigated agents. gZnO NPs had both of humeral immune suppressive effect mediated by Th2 rather than (direct) effect on B-lymphocytes and hence decline in the systemic level of Antibodies (Ab) In addition to suppressive effect on localized cellular innate effect Dexamethasone had direct immune- suppressive effect on B-lymphocytes, while less effect on Th2 pathway, hence the systemic level of Ab still elevated and represented by increase in total protein by the biochemical investigation. Dexamethasone (alone) suppresses the localized cellular innate response. This immune suppressive effect gets lost when admixed with gZnO NPs. Despite the eco-friendly nature of investigated gZnO NPs but it still had the genotoxic effect of ZnO NPs.

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