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The Effect Of Mgo Nps That Synthesis By *Streptococcus Spp*. On Immune System In Rabbits

[1]Yousif M. Jebur
[2]Frial G. Abd
[1,2] *Department of Ecology and Pollution, Faculty of Science, University of Kufa/ Iraq*

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**Corresponding Author:*

Emails:

sci.frialgemeel@uobabylon.edu.iq

Abstract: *The current study describes a low-cost, environmentally friendly, repeatable magnesium oxide nanoparticle manufacturing process mediated by Streptococcus spp. The U.V. spectrophotometer, Atomic Force Microscopy (AFM), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and Field Emission Scanning Electron Microscopy (FE-SEM) were all used to characterize the nanoparticles. The crystallographic cubic pattern seen by the XRD indicates that the structure of nanoparticles is mostly crystalline. The characterization peaks and functional groups of MgO nanoparticles were identified using FTIR analysis. Furthermore, the study of MgO NPS produced by S. salivarius and S. mutans using field-emission scanning electron microscopy (FESEM) revealed that their average diameters were 49.85 nm and 66.15 nm, respectively. The findings of all immunological tests conducted in this investigation showed that MgO N.P.s produced by S. mutans and S. salivarius were substantially different from the control group (P 0.05) for systemic and mucosal antibodies, respectively.*

In comparison to the control group, which had a mean value of (10)(1), MgO N.P.s biosynthesized from S. mutans and S. salivarius generated titers with mean values (1280)(2560) and (128)(256), respectively. In contrast to the control group, where the mitotic index resulted in a mean value of (3.56), the findings for the mitotic index were (10.16)(7.83). LIF in the test groups (51.33)(44.1) were significant compared to the control group (102.5). The MgO N.P.s elicited cellular immunity (delayed type hypersensitivity), which led to an increase in the spleen weight index in both test groups compared to the control.

Keywords*: *Streptococcus spp. .MgO nanoparticles .Immune system. N.P.s.XRD,AFM

1. Introduction

Nanotechnology is a modern field of science that researches phenomena at atomic, molecular and macromolecular scales, delivered by coupling of nanotechnology and biotechnology. Nanotechnology includes the synthesis, characterization, design, production and application of structure by controlling size and shape at the nanometer scale and

applications of nanoparticles[1,2]. The immune system is a collection of cells, substances, and mechanisms that defend the skin, airways, intestines, and other organs from germs (organisms including bacteria, fungus, and parasites), viruses, cancerous cells, and poisons. Innate immunity and adaptive immunity may be conceptualized as the immune system's two (lines of defense) simply [3]. There are two main reasons why understanding how nanoparticles (N.P.s) interact with the immune system and the potential for the induction of inflammation is crucial: first, it will help us understand potential health risks from N.P.s and, second, how the immune system detects and eliminates foreign substances. N.P.s will aid in developing medical nanoscale products (such drug delivery systems) capable of altering immune responses and evading immunomodulatory surveillance, enabling more effective treatment methods. An immune response that N.P.s may cause might destroy the alien components. The responder cells principally involved in this reaction are phagocytic and polymorphonuclear leukocytes (PMN), which eliminate N.P.s via phagocytosis and destruction [4]. Once swallowed, N.P.s are foreign substances removed mainly by macrophages, especially M2 macrophages. They could work well to encourage macrophage polarization, differentiation, or activation. The capacity of N.P.s to directly polarize macrophages to M1 or M2 and the propensity of N.P.s to change the phenotype from M1 to M2 and vice versa, either directly or by the delivery of cargoes, may be considered when evaluating how N.P.s alter macrophage polarizations[4]. N.P.s frequently congregate at the microscopic level, where macrophages can recognize them quickly and normally pick them up with ease. Larger NPs are digested more efficiently than smaller N.P.s. N.P.s can experience considerable surface modifications when they touch bodily fluids. Proteins and other plasma molecules may be absorbed by N.P.s, which can do so more readily with hydrophobic particles than with hydrophilic ones. The kind of absorbed protein depends on the chemistry of the N.P. surface and the water[5].

According to growing experimental evidence, innate immune system cells may interact with metal nanoparticles using the same techniques employed to destroy viruses. Critical sensitivity molecules known as toll-like receptors (TLRs) recognize conserved chemical patterns in bacteria and viruses and trigger an innate immune response [6]. CoNPs appear to be a sensor and signal transducer for the Toll-like receptors 4 (TRL-4), which activates the innate immune response and produces proinflammatory cytokines[7]. Inflammation may have a role in the toxicity of nanomaterials, which is why proinflammatory cytokines are frequently tested to anticipate immunomodulatory effects. Additionally, they can alter how effectively active medicinal components work when they are administered using nanomaterials [8]. One of the primary proinflammatory cytokines, IL-1b, is secreted due to the stimulation of NLRP3 inflammatory particle nanoparticles. Double-walled carbon nanotubes, for instance, enhance IL-1b, a proinflammatory cytokine secreted by human monocytes through the NLRP3 inflammatory pathway. Human monocytes were shown to produce IL-1b when exposed to silver nanoparticles, and cytokines released more effectively when particle size was decreased. Inflammation brought on by the silver nanoparticles was observed to release IL-1b [9,10]. The dermal cells and Langerhans cells in the skin's epidermis are examples of immunologically active tissues. Numerous studies shown that when injected, tiny (50 nm) nanoparticles are disseminated under the skin via the lymphatic system and the lymph nodes' outflow, where they activate lymphocytes and antigen-presenting cells. Lymph drainage is thought to enhance the adaptive immune response through a number of mechanisms, including local complement activation and T-cell stimulation [11]. Levels of proinflammatory cytokines were higher in the

tumor tissue from treated mice carrying B16 melanomas than in the control group (mice were immunized with empty nanoparticles), according to a study [12].

2. Methodology

Biosynthesis method of MgO N.P.s by *Streptococcus spp.*

Two isolates were used in the biosynthesis of MgO N.P.s *S. Mutans* was taken from addicted with (benzodiazepines and alcohol) and *S. Salivarius* was taken from no addicated person.At 37 °C for 24 hours, the bacteria were grown in N.B. After that, sterile, uninoculated N.B. was diluted with the bacterial cultures in a further 1:3 ratio. after dilution. Each received a 0.1 M addition of magnesium nitrate (Mg(NO3)2 6H2O). To slow the transformation and color change process, diluted culture was added drop by drop, followed by 0.2 M NaOH.Once the white colored precipitate had settled, the cultures were placed in a water bath at 40 °C for another 15-20 minutes. Finally, the cultures were incubated undisturbed for 10 hours at room temperature. The cultures were centrifuged at 5000 rpm for 15 minutes following a 10-hour incubation period. After carefully removing the supernatant, two washes with distilled water were performed on the particle. In order to acquire the nanoparticles in powder form, they were properly dried next. The generated nanoparticles were initially in the hydroxide form; however, by calcinating the nanoparticles at 300 °C for 4 hours, the hydroxide form was converted to the oxide form [13].

The generated MgO N.P.s were examined using FTIR, X-ray diffraction, field emission scanning electron microscopy, atomic force microscopy (AFM), and UV-visible spectroscopy.

Preparation of MgO N.P.s solution

MgO N.P.s were produced at a 4 mg/ml concentration by dissolving 4 mg of the nanoparticles in 1 ml of deionized distilled water, thoroughly combining them with a vortex, depositing them in an ultrasonic bath, and filtering the resulting mixture through a microfilter [14].

Lab Animals

A 9 adult males were employed. Newzland rabbits (Oryctylagus conniculus), aged 3 to 5 months, were used to measure the immunological response to an antigen. It was housed in cages designed for animals in a laboratory animal home and left there for two weeks to acclimate with the thought of using clean food and water for animals during the duration of studies [15].

Table(1): Experiment Design and Injection Method

Groups	Groups of animals MgO N.P.s (*S. salivarius*)	Groups of animals MgO N.P.s (*S. mutans*)	Groups of animals control
NO. of animals	3	3	3
Injected MgO N.P.s	MgO NPs (*S. salivarius*)	MgO N.P.s (*S. mutans*)	Normal saline
Dose amount	1ml for each Kg	1 ml for each Kg	1 ml for each Kg
concentration	4 mg/ml	4 mg/ml	0
No. of doses per week	3 weeks	3 weeks	3 weeks
Injection method per ml	0.25ml right muscle	0.25ml right muscle	0.25ml right muscle
	0.25ml left muscle	0.25ml left muscle	0.25ml left muscle
	0.5 ml subcutaneous	0.5 ml subcutaneous	0.5 ml subcutaneous

Immunization Program

A method (Al-Qas,2000)[16] was used in the immunization program, where 9 rabbits were used in the experiment, where 3 rabbits were injected with MgO N.P.s (*S. salivarius*) and also 3 rabbits were injected with MgO N.P.s (*S. mutans*) and the concentration was 4 mg/ml and an amount of 1 ml per Kg of rabbit weight. The control group injected 3 rabbits with Normal Saline, and the injection lasted 3 weeks, one dose per week.

The animals were dissected after being anesthetized with chloroform, blood was drawn directly from the heart (heart puncture) and placed in test tubes with anticoagulant and in tubes without anticoagulant to obtain the serum, and the appendix was taken to separate

the immunoglobulins and the spleen for spleen weight index test and also extracted from the thigh bone for a cell division coefficient test [17].

Immunological Tests

Secretory immunoglobulin extraction from appendix

The appendix was placed in a petri dish with normal saline, opened lengthwise with scissors, and washed with saline. 2- Sweep the mucous layer, suspend with 10ml of normal saline , and centrifuge at 3500 rpm/min for 30 min.3- The supernatant is mixed with an equal volume of PEG 6%, left at room temperature for 30 minutes, and then centrifuged at 3500 rpm / min for 30 min.4- The supernatant was discarded, and then the residue was suspended with 1 ml of normal saline [18].

Agglutination Test for Serum

1- Ten clean and sterile glass tubes were used, and 0.9 ml of normal saline was placed in the first tube and 0.5 ml in the remaining eight tubes by using clean and sterile mechanical pipettes. [19]

2- The volume of 0.1 ml of serum was added to the first tube and mixed well using clean, sterile pipettes.

3- A volume of 0.5 ml was transferred from the first tube to the second tube and from there to the next tube after mixing well each time and so for tube number 9 where it is transferred from a volume of 0.5 ml and discarded to be the dilution sequence 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280 , 1:2560 (Leave tube 10 as control tube).

4- A volume of 0.5 ml MgO Nanoparticles (according to required concentration of 4mg/ml) was added to each of the ten tubes.

5- The tubes were well mixed and incubated at 37 ° C for 24 hr.

6- The results were read through a cloudy mass, indicating the interaction of the antibody with the antigen .

7- The results were recorded by determining the titer, which means (inverted the highest dilution gave a positive result) .

Agglutination Test for Secretory Immunoglobulin

Ten clean and sterile glass tubes were used, and 0.2 ml of normal saline was placed in the first tube and 0.5 ml in the remaining nine tubes by using clean and sterile mechanical pipettes. [20]

2- The 0.2 ml of antibody suspension volume was added to the first tube and mixed well using clean, sterile pipettes.

3- A volume of 0.2 ml was transferred from the first tube to the second tube and from there to the next tube after mixing well each time, and so for tube number 9 where it is transferred from a volume of 0.2 ml and discarded to be the dilution sequence 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 (Leave tube 10 as control tube).

4- A volume of 0.2 ml MgO N.P.s (according to required concentration of 4mg/ml) was added to each of the ten tubes.

5- The tubes were well mixed and incubated at 37 ° C for 24 hours.

6- The results were read through a cloudy mass, indicating the interaction of the antibody with the antigen.

7- The results were recorded by determining the titer, which means (inverted the highest dilution gave a positive result) .

Mitotic Index Test

1- The animals were injected with 1ml of colchicine intraperitoneal and left for one hour and a half. [21]

2. Anesthetizing the animal with chloroform and placing it on its back and dissecting it by cutting the skin under the abdomen and pulling the skin to show internal guts.

3. Using a disposable wooden stick and a test tube filled with normal saline solution (5 mL), the femur's cellular contents were extracted after being cut off at both ends.

4. After carefully pipetting the suspended cell using a Pasteur pipette, the tube was centrifuged (at 2000 rpm) for 10 minutes.

5. The supernatant was discarded, the cell deposition was suspended in 5 ml of PBS, and the tube was then incubated in a water bath (37 ° C) for 30 minutes with gentle shaking every five minutes.
6. The tube was centrifuged for 10 minutes at 2000 rpm without the supernatant.
7. Each tube was filled with 5 ml of fixation solution, which was then gently mixed into the cell deposition to create a homogenous cell suspension. The tubes were then incubated at 4 ° C for 30 minutes.
8. After completing the previous procedure twice, the tube was centrifuged at 2000 rpm for 10 minutes.
9. From a height of about 2 feet, 4-5 drops of the cell suspension were placed onto a clean slide after the cell deposit had been well suspended in 2 mL of fixative solution.
10. The slide was cleaned with distilled water after being stained for 15 minutes with Gimesa stain and then air dried at room temperature.
11. The slide was examined under an oil lens (100 ×) and at least 1000 cells were examined for mitotic index.
The percentage of dividing cells was recorded using **The Mitotic index (%) = number of dividing cells/total count x 100**

Spleen Weight Index
1- Animals were anaesthetized with chloroform and dissected for spleen extraction. [22]
2- Removed the connective tissue from the spleen and placed it in a sterile petri dish containing PBS.
3- Dried the spleen on sterile blotting paper, then weight the spleen to calculate the spleen weight index, which is expressed as the percentage of its weight to body weight according to the following law:

$$\text{Spleen weight index} = \sqrt{\frac{spleen\ weight}{animal\ weight}} \times 100$$

Skin Test
This test was conducted in the fourth week, when each group of injected animals was injected with 0.1ml MgO N.P.s under or between layers of the skin. As for the control animals were injected with normal saline and the same amount, recording the observed skin change after (4,24,48,72) hr. compared to the control animals. Use this test to determine the effect of MgO N.P.s on sensitivity and their effect on stimulating the cellular response of rabbits [23].

3.Results and discussion

Characterization of MgO N.P.s

The U.V. spectrophotometer was used to characterize the nanoparticles. The MgO N.P.s' absorbance was measured throughout the range of wavelengths from 300 to 900 nm, and it was discovered that two isolates had greater absorbance at 400 nm. AFM (Atomic Force Microscopy) was used to The MgO N.P.s sample that was synthesized by isolate (S. mutans) had a root mean square roughness of 5.932 nm and 4.534 nm, respectively, while sample (S. salivarius) had a roughness of 7.400 nm and 5.461 nm, respectively. It was observed that the sample (S. salivarius) had a rougher surface than the sample (S. mutans). X-ray diffraction (XRD) revealed the cubic crystal structure of the produced MgO. The MgO N.P.s sample made by isolation S. salivarius had an average size of (34.23 nm), which was smaller than the MgO N.P.s sample made by isolate S. mutans, which had an average size of (41.79 nm)., Fourier transform infrared spectroscopy was used to identify the MgO N.P.s' functional groups. It was discovered that the functional groups (methyl and alcohol) had two peaks in the sample of S. mutans-produced MgO N.P.s while the functional group (alcohol and hydroxyl compound) only had one peak. Additionally, the MgO NPS generated by S. salivarius and S. mutans had average diameters of 49.85 nm and 66.15 nm, respectively, according to a Field-Emission Scanning Electron Microscopy (FESEM) investigation. Additionally, the MgO NPS generated by S. salivarius and S. mutans had average diameters of 49.85 nm and 66.15 nm, respectively, according to a Field-Emission Scanning Electron Microscopy (FESEM) investigation.

Immunological Tests

Humoral Immunity (**Systemic Humoral Immunity**)

The results of the tube agglutination test performed on test animals given MgO N.P.s (three animals per group) revealed a systemic antibody with mean value (2560) for MgO N.P.s produced by isolate (S. salivarius) and mean value (1280) for MgO N.P.s produced by isolate (S. mutans), while the test animals used as controls (three animals) produced a systemic antibody with mean value (10).

Table(2):Systemic antibody titer in serum of Rabbits group immunized with MgO N.P.s

Rabbits groups	NO. of animals	Type of MgO N.P.s	Systemic antibody titer mean
1	3	MgO NPs(*S. salivarius*)	2560"
2	3	MgO NPs(*S. mutans*)	1280"
3	3	control	10

" *Means a significant difference*

local Humoral Immunity

This study included the identification of local humoral immunity following the isolation of secretory immunoglobulins from the appendix from the groups treated with MgO N.P.s. The results showed high titer compared to the control, reaching (256) for MgO N.P.s that synthesis by isolate (S. salivarius) and (128) for MgO N.P.s that synthesis by isolate *(S. mutans).*

Table(3):mucosal antibody titer in appendix of Rabbits group immunized with MgO N.P.s

Rabbits groups	NO. of animals	Type of MgO N.P.s	Systemic antibody titer mean
1	3	MgO NPs(*S. salivarius*)	256"
2	3	MgO NPs(*S. mutans*)	128"
3	3	control	1

" Means a significant difference

Animals inoculated with MgO N.P.s underwent direct tests for agglutination in a tube containing animal serum and appendix globulin to identify the immune response (systemic and local) specific to N.P.s. These findings were compared to those of control animals.

According to Table (2) of the findings of our current investigation, two types of MgO N.P.s vaccinated mice had higher systemic antibody titers than control animals. Additionally, Table 3 demonstrated that animals exposed to two types of MgO N.P.s had mucosal antibody titers greater than those of the control group.

In order to promote innate or adaptive immunity, nanoparticles are tested for their immunostimulatory properties. The blood circulation's antigen-presenting cells, such as B cells, macrophages, and dendritic cells, are where nanoparticles often interact [24].These findings showed that MgO nanoparticles had the capacity to induce B cells to generate specific antibodies; this may imply that they can both initiate an immune response (immunogen) and interact with the byproducts of that reaction (antigen). These outcomes were in line with Park et al.'s findings from 2010 [25], who employed mice and silver nanoparticles to study the stimulation of an increase in the distribution of B cells.

Cellular Immune Response(Mitotic Index)

The results shown in table 4 showed a significantly higher rate of mitotic index of bone marrow cells for the group of animals treated with MgO N.P.s that synthesis by isolate (S. salivarius) and with a rate of (10.16) as well as for animals treated with MgO N.P.s that synthesis by isolate (S. mutans) and with a rate of (7.83) when compared with control animals, which showed a rate of (3.56) at P 0.05.

Table(4):The mitotic index of immunized rabbet groups with MgO N.P.s

Type of MgO N.P.s	No. of animals	Mitotic index Mean±S.D
MgO NPs(*S. salivarius*)	3	10.16"±1.040
MgO NPs(*S. mutans*)	3	7.83"±0.381
control	3	3.56±0.160

" *Means a significant difference*

It is necessary because immune cells mature at a set age inside the immune system. They are continuously being replaced by new cells, and these new cells come from immature cells in the bone marrow that will develop into the production of blood cells, hematopoietic stem cells in the bone marrow that have the capacity to regenerate, differentiate, and mature, and the process that regulates a number of factors the well-known soluble cellular cytokine in addition to hormones [26].The findings above showed that the nanoparticles could trigger an increase in cell division in the bone marrow, which is involved in the development of the body's immune cells. This may point to the MgO N.P.s' capacity to promote cellular immunity. According to other research, silver nanoparticles significantly enhanced the mitotic index and promoted cell division, which impacted the generation of lymphocytes[27,28]. Their contact with immune system components caused this effect.

Spleen Weight Index

Spleen weight index increased in rabbits immunized groups with MgO N.P.s that synthesis by isolate (S. salivarius) and MgO N.P.s that synthesis by isolate (S. mutans), respectively, at mean values (0.2490.157) and (0.2270.009) compared with control group (0.1180.008, table (5), and this increase was significant at P0.05.

Table (5): Spleen weight index

Type of MgO N.P.s	No. of animals	Mean±S.D	P value
MgO NPs(*S. salivarius*)	3	0.249"± 0.157	P≤0.05
MgO NPs(*S. mutans*)	3	0.227"± 0.009	
control	3	0.118± 0.008	

" Means a significant difference

The spleen is the biggest secondary lymphoid organ in the body, and as such, in addition to its duties in hematopoiesis and red blood cell clearance, it performs a variety of immunological tasks. The spleen's physical structure enables it to filter blood of infections and aberrant cells and promote connections between antigen-presenting cells (APCs) and cognate lymphocytes that are less likely to occur. These T and B cell responses to these antigenic antigens in the blood are regulated by splenic APCs [29]. Studies on the pathogenicity of various bacteria commonly use the impact of experimental infections on the shape and size of the spleen. The spleen-to-body weight ratios are given particular consideration in several vaccination potency studies, which have also embraced this phenomenon [30, 31]. In their investigation of the impact of gold nanoparticles on the liver and spleen, Balasubramanian et al. (2010)[32] discovered that the spleen retained more gold than the other organs during the course of the injection. Regardless of their size, shape, dosage, or kind of substance, injected N.P.s are stored for extended periods of time in the spleen.

Skin Test

This experiment was carried out to examine how MgO N.P.s affected the cellular immunity and cutaneous sensitivity of rabbits. Erythema, pus cells, induration, and necrosis were all visible. After 4 hours, the induration diameter of the animal groups that had received MgO N.P.s produced by the isolates S. salivarius and

S. mutans was 1.33mm and 1.33mm, respectively.

The skin of the rabbit groups that received MgO N.P. injections had pus cells and induration diameters of 10.66 mm and 9.66 mm, respectively, after 24 hours of injection. This was in comparison to the mean induration diameter after 4 hours, which had increased significantly at P 0.05.The findings also demonstrated that after 48 hours of injection, the skin of rabbits immunized with MgO N.P.s produced by isolates (S. salivarius) and (S. mutans) had developed pus cells and necrosis, with mean induration diameters of 12.66 mm and 10.4 mm, respectively, significantly different from those measured after 24 hours.

After 72 hours of injection, rabbit skin immunized with MgO N.P.s produced by isolates (S. *salivarius*) and (*S. mutans*) displayed erythema, pus cells, and necrosis. Additionally, the mean induration diameter had significantly decreased (8 mm, 6.66 mm, respectively) when compared to the mean induration diameter after 48 hours, with a P value of 0.05.

Table (6): Skin sensitivity of the immunized rabbits with MgO N.P.s

Type of MgO NPs	Mean±S.D Measure in mm			
Time of result	After 4 hr.	After 24 hr.	After 48 hr.	After 72 hr.
MgO NPs(*S. salivarius*)	1.33"±0.57	10.66"±2.08	12.66"±2.08	8"±1.73
MgO NPs(*S. mutans*)	1.33"±0.57	9.66"±2.08	10.4"±1.73	6.66"±1.15
control	00.00	00.00	00.00	00.00

" Means a significant difference

The DTH response, which can identify specific antigens to which the host has already had an immune response and provides an index of the current T-cell reactivity to specific recall antigens, requires prior immune sensitivity to a specific antigen and is therefore classified as a recall, or memory T cell response. The fact of redness and occasionally the degree of swelling that can be measured. to offer a DTH reactivity index [33].

Neutrophils are the main immune system players in humans with delayed type hypersensitivity (DTH), followed by a combination of macrophages and T cells. In contrast, in mice, neutrophils predominate in the mouse immune system to DIH antigens [34]. The CD8+T, CD4+T, and macrophage subsets of interstitial cellular infiltrates are predominant. The CD4 T-cells produce gamma interferon while promoting a cytokine cascade that activates nearby vascular endothelial cells, which promotes the rapid recruitment of neutrophils and subsequent additional macrophages and T-cell cytokines and induces delayed type hypersensitivity (DTH) reactions. Th1 cells also secrete IL-2, in addition to IFN-y, IL-2 and INF-y also induce the development of THI cells. Hydrolytic enzymes, reactive oxygen intermediates, and nitric oxide all play a role in the DTH process that causes tissue damage [35]. This leukocyte infiltration process disrupts the architecture of the local tissue and causes the redness and swelling that are typical of cutaneous DTH reactions [33].

These findings are consistent with other research [28,36] that demonstrated that using Ag NPs in rabbits to promote Th1 responses, which are primarily responsible for inducing proinflammatory cytokines, might result in delayed type hypersensitivity.

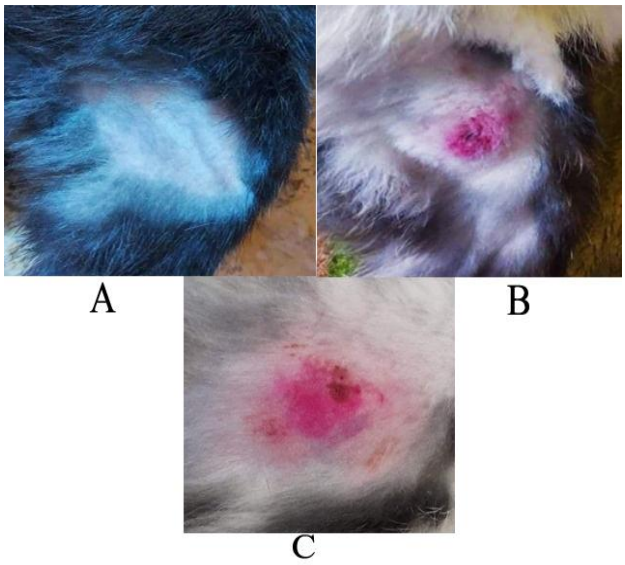

Figure (4-17): Skin test. A (control), B (MgO NPs(*S. mutans*)),
C (MgO NPs(*S. salivarius*))

4.Conclusion

MgO N.P.s are immunogenic and stimulate cellular and humoral immunity. The immune stimulation and the interaction of the particles with the immune system are both influenced by the size and roughness of the MgO N.P.s, as it was discovered from the results that the smaller the size and the rougher the surface of the MgO N.P.s, the greater the immune stimulation.

Ethical Approval

The specimens of this study took the patient's approval for adult patients, precious and the consent of the irrigation for young people in age as the law and directives of the human rights organizations with adequate information in an ethical manner

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