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Congenital Toxoplasmosis: Prevention of Vertical Disease Transmission by Vaccination

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Abstract. The efficacies of four different types of microwaved *Toxoplasma gondii* vaccines were evaluated for their ability to elicit protection in Swiss strain pregnant mice and their pups. Protection of dams and their pups was evaluated by parasitological and pathological studies. Mice were divided into 4 groups. Preimmunization of pregnant mice was done before mating. The first group immunized with tachyzoite vaccine alone, the second with tachyzoite vaccine in combination with cytokine IL_{-12}, the third with microwaved cyst vaccine alone and the fourth group with microwaved cyst vaccine combined with cholera toxin. All groups were challenged with the virulent RH strain of *Toxoplasma gondii* between the 10^{th} and 14^{th} day of gestation. The results showed highest level of protection in dams vaccinated with cyst vaccine in combination with cholera toxin followed by vaccine group in association with the cytokine IL_{12}. The combination of the vaccines with the adjuvant strengthened the vaccine effect, thus documented by the improvement of histopathological changes in the organs of both dams and pups compared to the infected control group organs. The study proposes the implication of this vaccine against the infection with *Toxoplasma gonidii*, not only in mice, but also in other mammalian hosts including women.

Keywords: Toxoplasmosis, MWI Vaccine, IL_{-12} Adjuvant.

Introduction

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan parasite that causes a variety of clinical syndromes, but the infection is severe in immune compromised individuals and during pregnancy due to the possibility of transplacental transmission of the parasite causing

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congenital toxoplasmosis. Vertical transmission of the parasite usually occurs when females are primarily infected during pregnancy. Infection also occurs orally through the ingestion of meat containing cysts or by the intake of food or water contaminated with oocysts[1-2].

The maternal immune response of women infected with *T. gondii,* for the first time during pregnancy appears to protect the mother but not the fetus. In fact, according to previous studies, it has been shown that cell mediated immune response is depressed during pregnancy[3-4]. Although numerous trials were conducted to study vaccine immunization for protection against toxoplasmosis, little is known about the vaccine immunization for protection against congenital toxoplasmosis. The trial of vaccination with live attenuated *T. gondii* proved to limit abortion and vertical disease transmission (VDT) during pregnancy[5]. Furthermore, vaccination with surface tachyzoite antigen (STAg) incorporated into liposomes did prevent fetal death and reduced significantly congenital infection[6].

Various types of irradiation (ɣ, UV, X-ray) were used experimentally to produce a killed or an attenuated vaccine/s against toxoplasmosis. It was shown that Microwave irradiation (MWI) has lethal effect on helminthes and protozoa parasites[7,8], hence application of MWI in producing immunogenic preparation was ventured[7]. However, possible application of (MWI) in preparing a vaccine against congenital or acquired toxoplasmosis was not tested previously. Accordingly, this study was carried out to test the possible immunizing capacity elicited by microwave irradiated *T. gondii* vaccines in pregnant mice experimentally infected with *T. gondii*. In addition, the effect of the immunostimulant cytokine (IL_{-12}) and cholera toxin (CT) was tested as vaccine adjuvant.

Material and Methods

Female Swiss albino mice were used for this experimental study, they were maintained to breed under conventional laboratory condition in our laboratory.

Vaccine Preparation & Dosage

The following parasitic strains were used to prepare the microwaved irradiated vaccines[7]. RH-strain of *T. gondii* was used to prepare microwave tachyzoites vaccine (MTV) and KSU-strain was used to prepare microwave bradyzoites cyst vaccine (MCV).

Infective tachyzoites of RH$^-$strains of *T. gondii* and bradyzoites cysts of KSU-strain, each separately was placed in a small pyrex container and exposed to the radiation in a Microwave (MW) oven (National N-N 9550-750 Watt-Matsushita Electric Industrial Co., Ltd., Japan) for fifteen seconds at 100% power[7]. Parallel preparations of non-MW irradiated parasites were used as controls.

The doses of immunization were determined according to a pilot study and were found to be as follows:

a) Microwave tachyzoites vaccine (MTV) was given to mice subcutaneously (S.C) in a dose of 0.05 ml or 50 µl (equivalent to 3 x 10^5 tachyzoites/dose) in three successive doses at two weeks intervals.

b) Microwave bradyzoites cyst vaccine (MCV) was given orally in a dose of 10^3/mouse and administered following the same schedule as MTV.

Adjuvants

Two types of adjuvant were selected for this study (IL_{-12} & Cholera Toxin) and administered with the prepared vaccines as follow:

IL_{-12} (sigma) was injected S.C to mice that received MTV vaccine and it was given for the same duration as the MTV was given, with a maximum cumulative dose of 4 µg/mouse[9]. While the Cholera toxin (Sigma) was administered to mice that received MCV vaccine, and it was given orally in a dose of 10 µg suspended in $NaHCO_3$ for the same duration the MCV was given[10,11].

Mice Grouping

Pregnant mice (dams) were designated into two clusters:

1. The control cluster of mice.
2. The experimental cluster of mice.

1. The **control cluster of mice** was arranged into three groups (10 mice/ group) as follows:
   - Control normal group that received nothing.
   - Control infected group that received only RH$^-$strains of *T. gondii*.
   - Control immunized group that received only the vaccine.

This group was further arranged into 4 subgroups and the subgroups were labeled as follow:

- Subgroup C1 received MTV vaccine alone
- Subgroup C2 received MTV vaccine + IL_{-12} adjuvant
- Subgroup C3 received MCV vaccine alone
- Subgroup C4 received MCV vaccine + Cholera toxin adjuvant

2. The experimental cluster of mice infected with *T. gondii* and received the vaccine.

The experimental cluster of mice was arranged into of 4 subgroups. The subgroups were challenged with RH^-strains of *T. gondii* and received the vaccine with or without adjuvant. The subgroups were labeled as follow:

- Subgroup E1 received MTV alone
- Subgroup E_2 received MTV + IL_{-12} adjuvant
- Subgroup E_3 received MCV alone
- Subgroup E_4 received MCV + cholera toxin adjuvant

Immunization and Challenge Procedure

Female virgin mice were immunized with either MTV or MCV with or without adjuvant as mentioned above. Immediately after the last dose of immunization, all groups of virgin mice were housed with males for seven days during which the females were examined for the presence of vaginal blugs and sperms in the vaginal lavage. The day the sperms were found in the vaginal lavage, it is the considered gestational day zero[12]. The dams were then allowed to bear their young normally. All dams of the experimental subgroups and the control infected group were challenged subcutaneously by 200 tachyzoites of RH^-strain/mouse on the day10-14 of gestation[13] (this is the minimal dose at which the mother mice is able to bear and deliver her young's successfully despite of being infected). Near full term (18-20 day), pups were removed performing Caesarian sections after killing the pregnant mice. Laboratomy was the preferred method of delivery instead of the vaginal delivered, and this to prevent the mothers from eating their congenital abnormal pups (anomalied pups). The uteri containing pups were removed intact; pups with their individual placentas were also removed. Before further handling, these materials were washed in tap water to destroy any viable parasite that might be present on the surface.

Evaluation of the Vaccine Efficacy

The vaccine effectiveness was assessed performing parasitological study and pathological examination of both the offspring (pups) and their Mothers.

1. Parasitological studies

The following parameters were measured for pups and mothers:

Pups	Mothers
I. Mean number of pups / each litter.	I. Number of mothers reached full term/group
II. Total number of living pups/ group.	II. Number of aborted mothers/ group.
III. Total number of dead pups/ group.	III. Number of mothers died before littering (reaching full term)/ group.
IV. Total number of pups with congenital anomalies/group.	IV. Health status of the placentas and uteri of mothers/group.
V. Vertical disease transmission (VDT)	

Detection of vertical disease transmission, in other words pups with congenital infection, was assessed by:

Examination of Blood Films stained with Giemsa, obtained from cord blood of pups to detect the presence of parasites in the cord or placental blood. Pups with positive parasitaemia were considered infected.

Negative pups, negative for parasites in the cord or placental blood were further evaluated by tissue and amniotic fluid Bioassay.

Bioassay of pups' tissue, each pup was killed and its organs (liver, spleen, placenta, lungs, heart, brain) were triturated together in normal saline and one ml suspension was inoculated intra-peritoneal into recipient mice. The recipient mice were then observed for developing peritoneal exudates containing the tachyzoites[14,15].

Bioassay of amniotic fluid, 0.5 ml of the amniotic fluid of each mouse was withdrawn and was inoculated into normal mice peritoneum. These mice were then evaluated as positive if developing peritoneal exudates containing the tachyzoites or negative if they do not[16].

2. Pathological examination

Pathological examinations of the pups and mothers were performed to detect gross and microscopic changes in the control infected group, control immunized subgroup and in all the experimental subgroups.

Pregnant mice were killed on the 18-20th day; their organs were removed and prepared for Pathological examination.

Pups were taken out and washed under tap water. On third of pups from each group were sacrificed just after birth, the second third where sacrificed one week after birth and the rest were sacrificed two weeks after birth.

The organs of each pup's group were removed, preserved and prepared for Pathological examination.

- The gross description of the congenital anomalies observed in the pups, as well as the gross pathological changes detected in the placentas and the uteri of the mothers were documented. Furthermore, the microscopic pathological examination of samples from the tissue organs of pups and mothers from control, and experimental clusters and groups was carried out on prepared slides stained with haematoxylin and eosin.

The immunohistochemical studies to aid in identifying the parasites were performed using strept-avidin-biotin-peroxidase complex staining technique[17] for antigen preparation and purification, and for preparation of the rabbit antisera (primary polyclonal antibodies).

- The control slides were prepared from normal mouse tissue (served as control for the staining reagents) and from infected mouse tissue, which received non-immune rabbit serum (this served as serum control).

Results

Parasitological Studies

Pups

The overall results shown in Tables 1 and 2, as well as in Fig. 1 and 2 revealed a significantly reduced mortality rate, VDT, congenital infection and congenital anomalies in all groups of pups originated from immunized challenged dames in comparison to the control infected group.

Table 1. Occurrence of toxoplasma transmission in pups borne from immunized pregnant mice with different toxoplasma vaccines after challenge with *T. gondii* RH⁻strain.

Groups	No. of pregnant mothers	Total No. of pups/Gr	Mean No. of pups / litter	Total No. of living pups/Gr	% (Survival rate)	Total No. of dead pups/Gr	% (death rate)	No. of pups with congenital anomalies/Gr	% (Rate of congenital anomalies)	Total No. of infected pups/Gr	% (VDT rate)
Normal control	10	82	8	81	98.8	1	1.2	0	0.0	0	0.0
Infected control	10	14	4	8	57.1	6	42.9	6	42.9	12	85.7
Gr E_1 MTV	10	28	5	20	71.4	8	28.6	3	10.7	13	46.4
Gr E_2 MTV+IL_{-12}	10	70	8	61	87.1	9	12.9	0	0.0	8	11.4
Gr E_3 MCV	10	44	6	36	81.0	8	18.0	0	0.0	10	22.7
Gr E_4 MCV+CT	10	87	9	82	94.2	5	5.7	0	0.0	3	3.4

Table 2. Protection after immunization with different toxoplasma vaccines of challenged pregnant mice with virulent RH⁻strain of *T. gondii.*

Groups	No. of mothers/Gr	No. of mothers reached full term/Gr	% (frequency of littering)	No. of aborted mothers/Gr	% (abortion rate)	No. of dead mothers/Gr	% (death rate)	No. of mothers with un-healthy uteri and placentas/Gr	%
Normal control	10	10	100.0	0	0.0	0	0.0	0	0.0
Infected control	10	4	40.0	4	40.0	2	20.0	5	50.0
Gr E_1 MTV	10	5	50.0	3	30.0	2	20.0	3	30.0
Gr E_2 MTV+IL_{-12}	10	9	90.0	1	10.0	0	0.0	0	0.0
Gr E_3 MCV	10	7	70.0	3	30.0	0	0.0	0	0.0
Gr E_4 MCV+CT	10	10	100.0	0	0.0	0	0.0	0	0.0

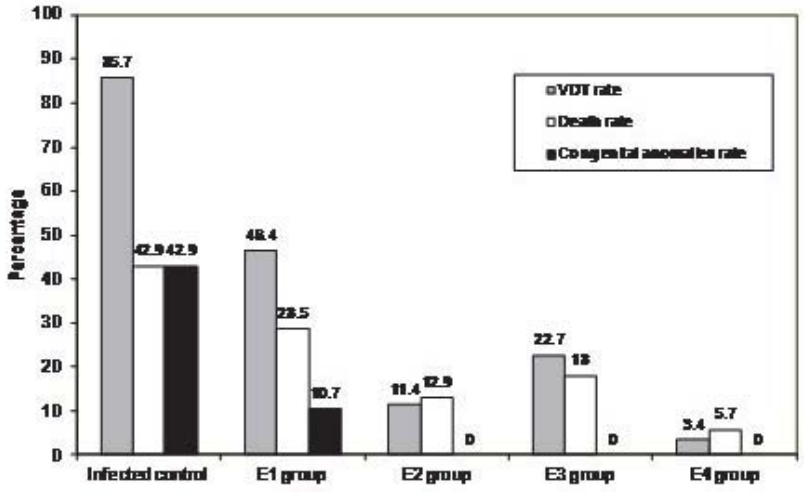

Fig. 1. Showing significant reduced Vertical disease transmission (VDT), Death rate & Congenital anomalies in all immunized challenged groups in comparison to the control infected group.

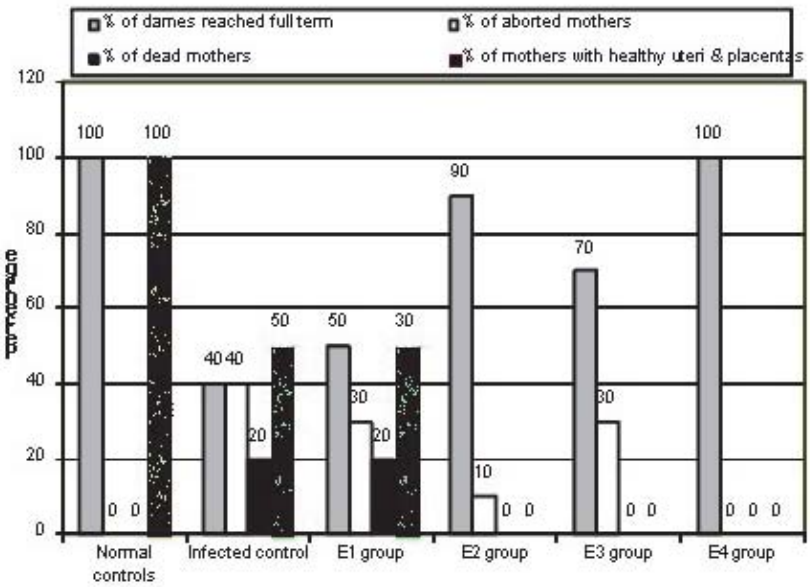

Fig. 2. Showing the percent of protection of the different toxoplasma vaccine immunization in control and experimental groups.

I. Mean Number of Pups / Litter (Litter Size).

The mean number of pups in each litter of the control infected group was significantly smaller (4 pups / litter) ($P<0.01$) than those expected from normal breeding stock mice (8 pups / litter). The mean numbers of pups in each litter in the experimental groups were (5, 8, 6, and 9) in E1, E2, E3 and E4, respectively.

II. Total Number of Living Pups/ Group (Pups Survival Rate).

III. The lowest survival rate was detected in the control infected group (57.1%). A higher survival rate was registered in pups of all the experimental subgroups E_1, E_2, E_3 and E_4 (71.4%, 87.1%, 81%, 94.2%), respectively. The E_4 subgroup showed the highest survival rate comparable to the control normal group 94.2%.

IV. Total Number of Dead Pups/Group (Pups Mortality Rate).

The highest rate of mortality was detected in the control infected group (42.9%). A much lower mortality rate (28.6%, 12.9%, 18.0% and 5.7%) was registered in pups of the experimental subgroups E_1, E_2, E_3 and E_4, respectively. The E_4 subgroup showed significantly lowest mortality rate than all other experimental groups ($P < 0.05$).

V. Congenital Anomalies.

Congenital anomalies were detected in the infected control group (42.9%) and in the experimental subgroup E1 (10.7%) that received the MTV vaccine alone. No anomalies were detected in the other subgroups E_2, E_3, E_4, which received different vaccines, MTV + IL12, MCV alone, and MCV + cholera toxin (CT), respectively.

VI. Vertical Disease Transmission (Infected Pups).

Congenital transmission occurred in all dames of the control infected group (infected with *T. gondii*) and in all the experimental subgroups (infected with *T. gondi* and received different vaccines). The highest rate of congenital infection was found in pups of the control infected group (85.7%). However, the percentages of vertical disease transmission diminished in all immunized experimental mice subgroups. Among the immunized groups, the lowest percentage of vertical disease transmission was noticed in pups born from dames of the subgroup E4 that was vaccinated with MCV+ CT (3.4%). The difference between all the experimental groups was statistically significant.

Mothers

VII. Number of Mothers Reaching Full Term/Group (Littering).

In the control infected group, out of 10 mothers, 4 mothers (40.0%) only littered. All mice of group E4 that received MCV in combination with CT reached full term. 50.0%, 90.0% and 70.0% of dames of subgroup E1, E2 and E3, respectively, completed their gestational periods.

VIII. Number of Aborted Mothers/Group (Abortion).

In the control infected group, 40.0% of mothers ended in abortion. Equal percentages of abortion (30.0%) were observed in dames vaccinated with MTV (E1 subgroup) and with MCV (E3 subgroup), while it was 10.0% in those mice received MTV with IL_{-12} (E2subgroup). No single case ended in abortion noticed in dames vaccinated with MCV in combination with CT (E4subgroup).

IX. Number of Mothers Died before Littering (Reach Full Term)/ Group (Death of Mother Mice).

Out of 10 mice, 20% of the mothers in the control infected group died before giving birth and they were found in post examination to have resorbed their embryos. 20.0% of dames of E1 subgroup that received MTV alone died before giving birth, while all dames of E2, E3 and E4 subgroups survived and gave birth at the end of the experiment.

Parasitological results of the control immunized dams, but not infected (C1, C2, C3 and C4 subgroups), and their pups revealed similar results to those encountered from the control normal pregnant mice and their pups. This indicates that the vaccination alone has no effect on the organs.

Pathological Results

A) Macroscopic Pathological Examination of Pups and of the Uteri and Placentas of Dams.

Macroscopic examination of the uteri and placentas of the mothers and macroscopic examination of the pups for congenital anomalies were performed.

The macroscopic examination of the uteri and placentas revealed unhealthy uteri and placentas in 50% of the control infected group, and 30.0% of the experimental subgroup E1 that received MTV vaccine alone. Uteri and placentas of mothers from the experimental subgroups E2, E3, and E4 were healthy and had no abnormal gross changes (Fig. 3D normal uterus and Fig. 3F1 normal pup and placenta). The unhealthy uteri and placentas exhibited inflammation and atrophy with multiple areas of congestion and necrosis (Fig. 3E abnormal uterus and Fig. 3F2 macerated atrophic placenta).

The macroscopic examinations of the congenital anomalies in pups were mainly in the form of hydrocephalus (Fig. 3B), microcephaly (Fig. 3C), complete maceration (Fig. 3F2) and vesiculation of the pups (Fig. 3F3).

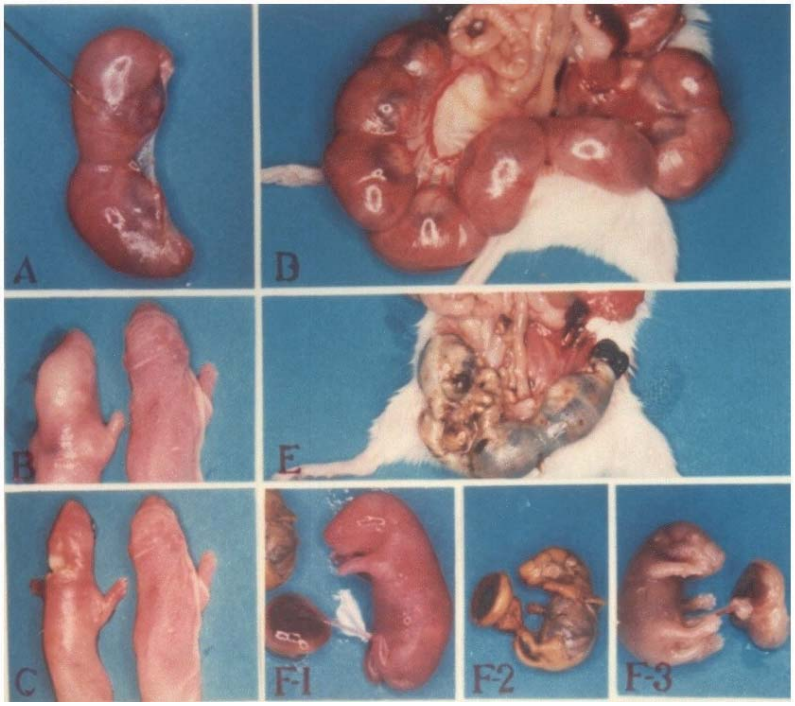

Fig. 3. A) Sample taken from the amniotic fluid; B) Pup showing hydrocephalus; C) Pup showing microcephaly; D) Control mother rate showing normal uterus; E) Infected mother rate showing abnormal uterus with areas of congestion and necrosis; F1) Normal pup showing normal placenta; F2) Congenitally infected pup showing macerations and atrophic placenta; F3) Congenitally infected pup showing vesiculation.

B) Microscopic Pathological Findings of Pups Organs at Birth and at One Week after Birth in the Control Infected Group and Experimental Subgroups.

Examination of pups' organs (brain, liver, heart, spleen, lymph-node, kidney, lung, and placenta and cord blood) just after birth revealed no histopathological changes in all groups of pups from the control infected group and experimental subgroups. One week after birth, only mild inflammatory infiltrate with mononuclear inflammatory cells was seen in the organs of pups'. No parasites could be detected in pups' organs at birth or one week after birth.

C) Microscopic Pathological Findings of Pups Organs Two Weeks after Birth in the Control Infected Group.

Ten percent (10%) of pups originated from the control infected group two weeks after birth were found to be free of parasitic infection and show only mild mononuclear inflammatory infiltrate. However, 90% of the pups, on the other hand, revealed severe histopathological changes in the pups' organs.

Brain: The brain showed perivascular lymphocytic infiltrate affecting the gray and white matter around the aqueduct and ventricles. Focal areas of necrosis with active gliosis were observed. (Fig. 4A).

Liver: The liver of these pups showed preserved architecture with prominent congestion of the central veins, thrombi formation and mononuclear inflammatory infiltrate. The hepatocytes showed degenerative changes ranged from hydropic degeneration to moderate steatosis, as well as regenerative changes including high mitotic activity and many binucleated cells (Fig. 4B-D). Multiple necrotic foci associated with polymorph nuclear leukocytes infiltrate, lymphocytes and macrophages were observed. Infrequent *T. gondii* tachyzoites were detected.

Cardiac Muscle Fibers: Showed hyaline degeneration, interstitial edema, congestion and mild infiltration by mononuclear inflammatory cells, mainly macrophages and lymphocytes (Fig. 4E).

Spleen: Showed mild hyperplasia of the white pulp with increased mitotic activity. The red pulp showed congestion and histiocytosis. Focal

myeloid metaplasia was evident. Few *T. gondii* tachszoites were identified in the H/E sections (Fig. 4F-H).

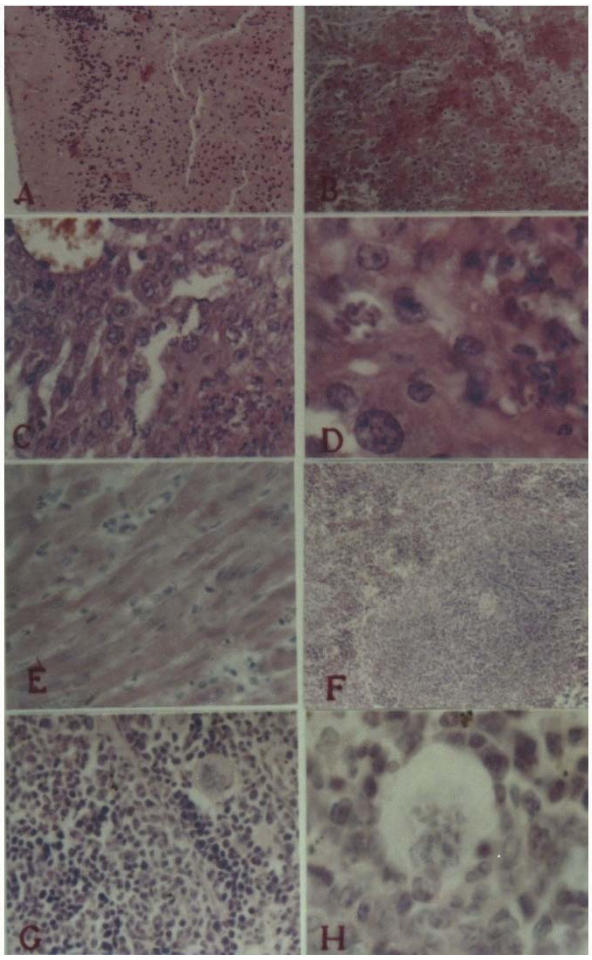

Fig. 4. Congenital toxoplasmosis: A) Brain showing focal mononuclear inflammatory infiltrate and areas of necrosis and gliosis (H&E x 100); B) Liver showing congestion and hydropic degeneration of liver cells (H&E x 100); C) Liver showing congestion, necrosis, inflammatory infiltrate of lymphocytes, plasma cells, and neutrophils. Mitotic figure and binucleated cells are seen (H&E x 400); D) Liver showing tachyzoits. (H&E x 1000); E) Cardiac muscle fibers showing hyaline degeneration, congestion and mononuclear inflammatory infiltrate (H&E x 100); F) Spleen showing hyperplasia of white pups (H&E x 100); G) Spleen showing myloid metaplasia (H&E x 400); H) Spleen showing tachyzoits (H&E x 1000).

Lymph-nodes: The generalized lymphadenopathy was associated histologically with follicular hyperplasia and large areas of necrosis

infiltrated by neutrophils. Epithelioid histiocytes aggregates forming non-caseating micro-granulomas encroaching upon the lymphoid follicles were also evident. No parasites could be identified in H&E stained sections (Fig. 5A, B).

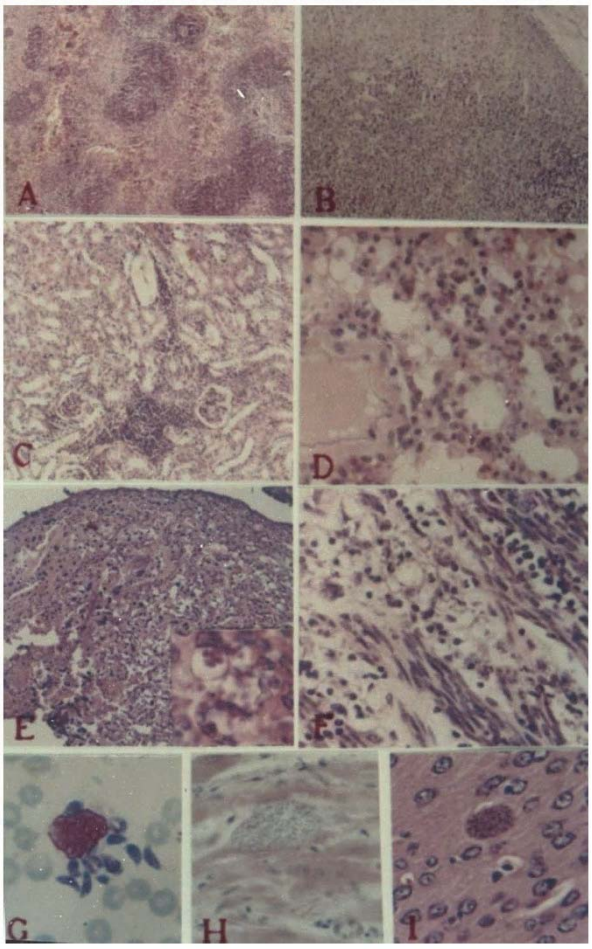

Fig. 5. A) Lymph node showing follicular hyperplasia and sinus histiocytosis (H&E x 100); B) Lymph node showing epithelioid histiocytes forming non caseating granuloma (H&E x 100); C) Kidney showing interstitial inflammatory infiltrate (H&E x 100); D) Lung showing congestion and interstitial pneumonia. (H&E x 400); E) Placenta showing trophoplastic hyperplasia and wide areas of necrosis (H&E x 100); inset showing tachyzoits (H&E x 1000); F) Maternal myometrium showing oedema, intense inflammatory infiltrate, and many tachyzoits . (H&E x 400); G-I) Cord blood showing ruptured macrophage releasing intracellular tachyzoits (Giemsa x 1000).

Kidneys: The pup's kidneys showed swelling of epithelial cells of proximal and distal convoluted tubules. Interstitial inflammatory infiltrate by mononuclear inflammatory cells, mainly lymphocytes and macrophages was present. The glomeruli showed no pathologic changes. No parasite could be identified in H/E stained sections (Fig. 5C).

Lung: Showed marked congestion with focal interstitial hemorrhage. Interstitial mononuclear inflammatory infiltrate was prominent in alveolar septa, peri-bronchial and connective tissue. No parasite was detected in H&E stained sections (Fig. 5D).

Placenta: The placental tissue revealed marked congestion, trophoblastic hyperplasia, focal hydropic degeneration and wide areas of necrosis. Few parasites are identified with difficulty in H&E stained sections (Fig. 5E).

Cord Blood: Samples from the cord blood, stained with Giemsa stain, revealed many tachyzoites phagocytosed by macrophages. Rupture of the cell membrane lead to free tachyzoites in the cord blood (Fig. 5G).

Immunohistochemical Staining: Assisted in the identification of the parasite, and in fact, more numbers of parasites were detected with this staining technique than with H& E stained sections. Tissue sections (Fig. 6) from various organs (including liver, spleen, lymph nodes, cardiac muscle fibers, lungs, placentas and uterine muscles) revealed positive staining confined to tachyzoites, in the inner wall of parasitophorous vacuole and in the cell membrane of the infected cells. No parasite could be demonstrated in the brain or the kidney of pups.

D) Microscopic Pathological Findings of Pups Organs 2 Weeks after Birth in the Experimental Subgroups.

Examination of organs of pups originated from the experimental subgroup E1 that received MTV alone revealed less severe histopathological microscopic changes when compared to the control infected group. Some pups' tissues were found free of parasite infection. Examination of organs of pups of the remaining experimental subgroups E2 and E3 revealed more or less normal preserved architecture with minimal inflammatory infiltrate, in the form of mononuclear inflammatory cells with very few numbers of tachyzoites in some cases. Organs of pups of the experimental E4 subgroup that received MCV and

CT showed normal preserved architecture with minimal inflammation, and were found free of parasite infection.

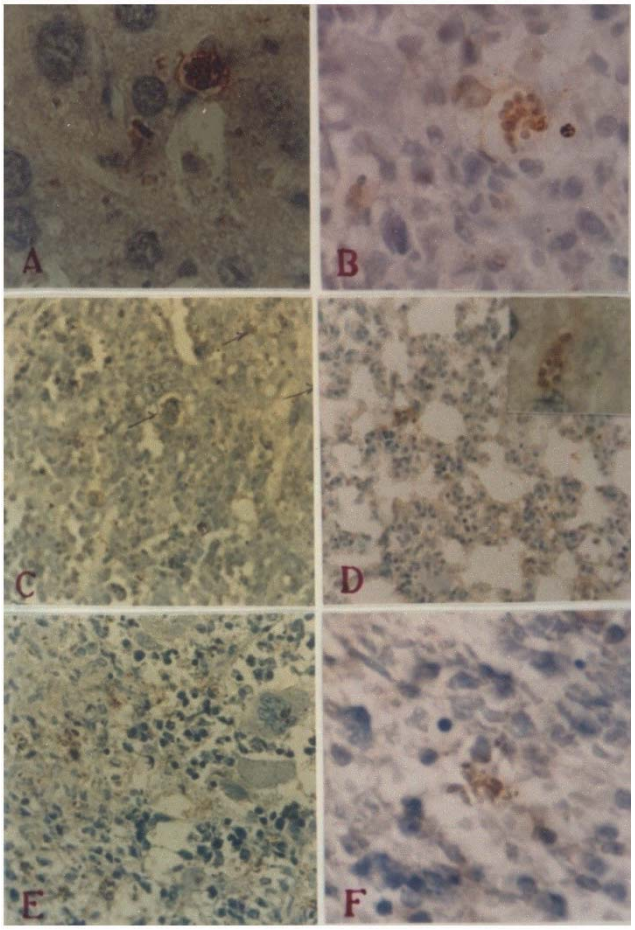

Fig. 6. Immunohistochemical staining showing positive tachyzoits in different pups' organs: A) Liver (immunostain x 1000); B) Spleen (immunostain x 1000); C) Lymph nodes (immunostain x 400); D) Lung (immunostain x 400, inset x 1000); E) Placenta (immunostain x 400); F) Uetrus (immunostain x 1000).

E) Microscopic Pathological Findings of Mothers Organs of the Control Infected Group.

Examination of the mothers' organs of the control infected group on 18-20th day after removal of the litters', revealed similar microscopic changes of the organs of the pups generated control infected mothers. Furthermore, the examination of maternal uterine muscle fibers of the

control infected group showed massive interstitial edema, congestion and infiltration by mononuclear inflammatory cells; mainly lymphocytes and histiocytes. Few tachyzoites were detected (Fig. 5F).

F) Microscopic Pathological Findings of Mothers Organs from the Experimental Subgroups.

Examination of dames organs of the subgroup E1, which received MTV alone were found to contain mild changes when compared to the mothers' control infected group, and with less number of tachyzoites. Some cases were found normal, except for minimal inflammatory cellular infiltrate. Microscopic changes of the dames' organs from the experimental subgroup E2 and E3 were showed milder changes when compared to the control infected group. The examination of the mothers' organs from the subgroup E4 that received MCV with CT revealed more or less normal preserved architecture. No pathological changes could be noticed except for minimal mononuclear inflammatory cells infiltrate and complete absence of tachyzoites.

An interesting finding was the observation of cystic stages of *T. gondii* parasite in the brain and cardiac muscles of some mice form the experimental subgroup E2 that received MTV with IL_{-12}. The cystic stages are very similar to the one encountered in mice infected with cyst forming strains of *T. gondii* (Fig 5H, I).

G) Microscopic Pathological Findings of Mothers and Pups' Organs from the Control Immunized Subgroups.

The microscopic examination of the control immunized non-infected subgroups (C_1, C_2, C_3 and C_4) mothers and their pups' organs demonstrated a picture similar to the control normal group tissues, except for minimal cellular infiltration by lymphocytes.

Discussion

A safe and effective vaccine against toxoplasmosis would be of interest due to substantial morbidity and mortality caused by *T. gondii* in congenitally infected infants and in immunocompromized individuals.

This study describes a defined vaccine that resulted in protection of both mother mice and their offspring's from the lethal challenge with the virulent *T. gondii* RH^{-}strain in experimentally infected model. The important feature in this study is the use of novel vaccines killed by a

new method of irradiation which is the Microwave irradiation (MWI). Microwave irradiation (MWI) has shown to induce lethal effect on helminthes and protozoa parasites[18]. However, this preparation was not tested previously for its possible application in producing immunogenic preparations to protect against *T. gondii*. Therefore, this study is the first to report the use of microwave irradiated *T. gondii* vaccines to protect against congenital toxoplasmosis.

The present study demonstrated that all mice dames that were immunized with the described vaccine revealed some degrees of protection. This protection varied according to whether the antigen was used alone or in association with its adjuvant. The highest level of protection seen in this study was observed in dames received MCV in combination with CT followed by the group received MTV in association with IL_{-12}. Although there is no significant difference between the protection offered by MTV and the infected control group in regard to the protection of the mothers, it has succeeded in the protection of pups against congenital toxoplasmosis transmission.

Many studies described the use of live and/or killed vaccines against murine toxoplasmosis[19-24]. Other studies reported the use of live chronic cyst forming strains, live attenuated strains or killed vaccine preparations for protection against congenital toxoplasmosis. However, little is known about the use of microwave irradiated (MWI) vaccines in protection against congenital toxoplasmosis.

Remington *et al*.[14] in 1961 and Roberts and Alexander[23] in 1992 used the live chronic cyst forming strains of *T. gondii* to induce immunity against vertical disease transmission (VDT) in mice. In the study of Remington *et al*.[14], pregnant mice were immunized before pregnancy by Beverley strain, and then challenged during pregnancy by RH^{-}strain. However, Robert and Alexander[23] reported the use of RRA strain for immunization of their mice and then challenged them by virulent yeast forming strain. The degree of VDT protection reported by Remington was 47.3% in mothers. In contrast, the VDT protection was 100% in Roberts and Alexander's study[23].

MacLoed *et al.*[5] used a live attenuated (temperature sensitive mutant strains of *T. gondii*) to immunize pregnant mice. They stated that this type of vaccination limits abortion and VDT during pregnancy, and that the range of protection in the neonates was very limited (36.0%). This

percent of protection contrasts with the present study finding, which encountered high levels of protection against VDT ranging between 53.6% to -96.6%. Furthermore, this study finding is more comparable with Roberts and Alexander's study finding[23].

Therefore, it is inferred that the difference in the degree of protection between the present study and the previous studies might be contributed to the use of different types of *T. gondii* strains, different vaccine preparation, and different routes of administration as well as different doses of immunization to challenge the infection. The second important point to be considered in this regard is the use of microwave irradiated *T. gondii* strains in preparing the vaccine. Contrasting the three previous studies were they used live vaccines, which also have a possible risk of recrudescence in the mother mice.

In fact the possibilities of recrudescence have drawn the attention of Roberts *et al*.[6] and El-Saied *et al.*[24] to the use of the killed vaccines in vaccinating pregnant mice. In addition, in trying to enhance the immunity of those killed vaccines they used different types of adjuvant (entrapping killed vaccines into liposomes[6-24]). An interesting result by Roberts *et al*.[6] was the finding that vaccination with surface tachyzoite antigen (STAg) without adjuvant increased the rate of fetal death of the neonate more than that observed in neonates from non vaccinated dames. El-Saied *et al.*[24] confirmed these findings. Roberts *et al*.[6] on the other hand, explicate their finding stating that this detrimental effect of killed vaccine (STAg) without adjuvant was probably due to the fact that STAg contained counter-protective as wells protective antigens. Furthermore, those antigens might be protective when they are enhanced by entrapping into liposomes (adjuvant) and lethal when they are introduced in their soluble form (without adjuvant).

The paradoxical effect encountered in the studies of Roberts *et al.*[6] and El-Saied *et al.*[24] contrasted the results of the present study that revealed significant levels of protection elicited when either MTV or MCV were used alone without adjuvant. The above studies raised an interesting point but they offered no documentation on the effect of their vaccine on the normal mice tissue. In other words, the lack of studying an immunized control group without parasite challenge, hinders a complete explanation on the real cause of lethality of the pups.

Moreover, the role of the use of different types of adjuvant, with the toxoplasmosis antigen, in augmenting the protection against acquired toxoplasmosis was investigated in many studies. Freund's complete adjuvant has been used successfully in a number of studies[25,26]. Administration of purified *T. gondii* encapsulated antigen form in liposome[27] or non-ionized surfactant vesicle adjuvant[25] were found to augment protection against acquired toxoplasmosis in mice. The present study further confirmed these findings found a high protection using MCV with TC adjuvant and MTV with IL-12. Recently, immunization with *E. coli* expressed recombinant SAG1 with IL_{12} has induced profound protection against lethal infection of *T. gondii* in mice[21].

Based on the established adjuvant role of the immunostimulent cytokine IL-12 in enhancing immunity against *Leishnania major*[28], *Mycobacterium tuberculosis*[29] and *Listeria monocytogenes*[30]. Therefore, IL-12 was chosen to enhance the immunity of our MWI killed tachyzoites vaccine (MTV). This protocol resulted in induction of significant level of protection against the challenge with the highly virulent RH^- strain in both in mother mice and their offsprings.

The mechanism of action of IL-12, like the natural IL-12 secreted by the monophages is the rapid and strong orientation of the immune response towards the activation of T-cells and NK-cells to produce IL-12 α-INF and the development of Th1 response[31,32]. α-INF in turn, activates macrophages toxoplasmicidal effect in the presence of a co-factor like TNF. Moreover, Th1 inducing immunoadjuvant effect of IL-12 has clearly been shown in several experimental models of infection[28,33,34].

Unexpectedly, however, the vaccinated mice (with MTV + IL-12) surviving challenging infection were found to harbor latent parasitic stage (cyst forms), which are not normally encountered during infection with RH^- strain of *T. gondii*. The reports on cyst formation by the virulent RH^- strain revealed that cyst formation was dedicated when the naturally resistant, host like rats are used, or when chemotherapeutic agents are used, or in prior immunization[35-37]. In these investigations as well as in the current study, the cyst forms were infrequent in number and resemble the cysts of the a-virulent strains, and they were resistant to pepsin-HCl digestion. An important concern was entertained following the finding of latent parasitic forms in mice vaccination with MTV in

association with IL_{-12} is the risk of developing toxoplasmosis infection at any stage if there is a breakdown of latency for any reason. Such possible reversal to virulent form would make such vaccine undesirable for application in human.

The above observation underscored the need of developing another vaccination schedule that could enhance the sterile immunity, and thereby, eliminate the possibility of latent infection. This was fulfilled in the present study by the use of cholera toxin (CT) as an adjuvant to the microwave cyst vaccine (MCV). This method of vaccination was based on direct immunization of the mucosal surface and the MCV alone was used in conjugation with CT. The results indicated that CT has strengthened the immunogenecity of the MCV to the degree of offering complete protection of the dames, and a very high level of protection of pups. This potent effect could be explained in the view of the dual action of CT (enclose cellular and humoral immunity) as an adjuvant as it was shown in many studies[11-39]. These studies have indicated that immunization with CT could induce mucosal and systemic immune responses, and offer a long lasting resistance against the establishment of toxoplasmosis). Bourguine *et al.*[40] postulated another mechanism of action of the CT adjuvant, which is increasing the microbistatic activity of the peritoneal macrophages against toxoplasmosis, and increase the penetration power of the *T. gondii* antigens into the intestine epithelial cells (antigenic carrier). In addition, type I-pattern of response has also been entertained[40,41].

This report addressed a second point which is the use of (MWI) that resulted in production of toxoplasma vaccines characterized by significant protection of both dames and offsprings. Our offered explanation of this significant protection by these vaccines is that degradation of the parasites by MWI occurred. The subsequent release of toxoplasma antigens was as equally effective as those released by homogenization[7]. Moreover, it was shown that MW irradiation is extremely effective in killing of the parasites without exceedingly distorting them. In fact, it was proved that MWI has killing effect on Eimeria, Strongyloides, Tania tamifamis[7]. Bouchet *et al.*[42] considered and proposed the prophylactic use of MWI technique. In this study, it is assumed that the protections induced by the antigens, which are present on the surface of the parasite, were preserved.

The present study raised an interesting question, which is whether the heat generated during MWI is the solo responsible for the killing effect of the parasite during the process of vaccine production. There is evidence that thermal energy is not wholly responsible for MWI induced effect on the microorganisms[43,44], and hence, non-thermal microcidal effects of the MWI play a role[45,46]. Whether thermal or non-thermal or both were the effectors, MWI still provide advantages over the traditional methods of producing killed vaccines as it is easier, faster, cleaner and less expensive than other methods used in the production of vaccines.

Vaccines prepared from one strain of *T. gondii* have been shown to protect against another forms, despite the fact that tachyzoites, bradyzoites and oocysts express stage-specific antigen[5,27,47]. This study further confirmed these data, and documented that giving MWI tachyzoites RH^- strains or bradyzoites KSU strain of *T. gondii* were effective in protecting against the virulent RH^- strain challenge.

The determination of toxoplasmosis fetal infection was carried out in various studies by using ELISA technique[6,23]. The current study however, for determining the congenital toxoplasmosis infection in mice used three methods, including direct parasite determination in cord blood, bioassay of negative pups tissue and amniotic fluid samples, which certainly increased the sensitivity of the results.

Detection of the tachyzoites in cord blood alone is a sure indication of infection. The parasitaemia seen in the infection represents the presence of infective leukocytes rather than free organisms within the blood. It is postulated[15] that the mechanism by which the parasite transfers through the placenta is either by the wandering leukocytes through the cyncitial trophoblast into the fetal circulation, or by rupture of the parasitized white cells near the placenta allowing the liberated organisms to cross the membranes. The present study revealed high degree of parasitaemia in the cord blood together with the presence of the parasites in the placental tissue, which further supported Fux *et al.*[15] hypothesis and indicated that infection of the pups was either due to the presence of blood containing parasites or to the presence of aggregates of parasites in the placental tissue as well.

Another interesting finding of the present work is the demonstration of the organisms in the cord blood or by the bioassay methods just after their birth, while these organisms were not detected histopathologically

until the end of the second week after birth. These results were consistent with data which failed to demonstrate the parasites before the 9th of birth[14]. However, further investigation on the extent of proliferation of the parasite in the fetuses should be carried out in future studies, since data in this regard is scanty.

From the histopathological point of view, the current study is in agreement with others[48,49] that the basic tissue changes seen in the liver and spleen of congenitally infected mice consisted of presence of parasite in parynchymal cells and macrophages leading to cellular degenerative changes compiling up to sever focal necrosis, and inflammatory infiltrate with mononuclear cells and neutrophils. Formation of pseudo cysts containing tachyzoites was evident in H/E as well as in immunohistochemically stained sections. Inflammatory reaction caused by ruptured cysts, with superadded vasculities leading to necrosis in the corresponding areas. Eissa *et al*.[48] and Lee *et al.*[49] could not however detect the parasites in the tissues of congenitally infected neonates with the conventional staining methods, while Dutton *et al*.[50] did by using Immunohistochemical techniques to detect the parasite antigen. Eissa *et al*.[48] attributed these conflicting results to the virulent strain used in their study.

The focal areas of myeloid metaplasia detected in the spleen of the congenitally infected mice and reported in this current study was previously observed by others[48-51] during the first two months post inoculation. The myeloid metaplasia of the spleen was attributed to the infiltration of the bone marrow by the parasites and the inflammatory infiltrate.

In the present work, the brain involvement was similar to what was reported by others[48-52]. It revealed cortical perivascular inflammatory infiltrate, focal necrosis with gliosis and ventriculitis and the inflammatory response was predominantly mediated by lymphocytes. Furthermore, others described wide areas of inflammatory necrosis, circumscribed granulomatous inflammation, and pseudocysts containing a variable number of organisms, as well as free tachyzoites in damaged areas[53]. These findings indicated that the congenital murine *toxoplasma* infection was also closely parallel to human congenital toxoplasma encephalitis and differed significantly from adults' toxoplasmosis which

result in subcortical demyelization and multiple well circumscribed necrotizing abscesses[54].

The study of Benirschke *et al*.[55] showed that human toxoplasma resulted in placental changes consisted in chorionic villites. This observation was similar to what the present work showed, mainly trophoblastic hyperplasia, inflammation and degenerative changes in placentas of infected pups. Furthermore, he raised the attention to the possibility of easily overlooking the toxoplasma pathogens identification in routine placental study. On the other hand others studies emphasized the valuable role of chorionic villus sampling in the detection of congenital toxoplasmosis[56]. The visualization of the extent of *Toxoplasma* infection in the examined tissue in the current study was made possible by the immunoperoxidase staining without compromising the cellular details or histopathologic changes. In fact, *Toxoplasma* parasites were easily identified in lymph node and lung sections which were difficult to detect in conventionally stained sections. This finding emphasizes the importance of using this stain for identifying the *Toxoplasma* pathogen in future studies. Furthermore, in the current work, we showed that the staining is specific, since it was limited to the trophozoites, the inner wall of the parasitophorous vacuoles and to the cell membrane of the infected cells. This denotes that there were no diffuse antigens present in the tissue and the specificity of the staining could be attributed to the concentration of the antigen produced by toxoplasma parasite within the infected cells. However, it was reported rare rupture of the cyst wall and the presence of extracystic toxoplasma antigen (endozoites), which were identified by electron microscopy in the inner areas, surrounding the ruptured cyst[57].

In conclusion, the results of this study demonstrated that preimmunization with microwave irradiated (MWI) vaccines against *T. gondii* before pregnancy were immunogenic and protective for both, pregnant mice and their offsprings. Furthermore, it is advantages to use MCV in combination with CT to the mucosal immune system to evoke immunity in the intestinal tract and priming the immune system at more distant sites. The work did document that MCV with CT was immunogenic enough and produced complete protection of dames and diminished the rate of vertical disease transmission in pups. Another manifestation of the high degree of immunity and protection offered by this vaccine was evident by the complete absence of pathological

changes in both dames and pups organs. Moreover, the application of the immunoperoxidase technique to identify the parasite would pave the way for direct, easy, and reliable diagnosis of *T. gondii,* especially in lymph node biopsy and tissue of aborted human fetuses.

An important point to investigate is if MCV in combination with CT is safe during pregnancy to determine its application to vaccine, in both human and animals. Another recommendation drawn from this work is the need for future studies to determine if microwave killed or attenuated prepared vaccines may be effective against other parasites. If so, the MWI may provide a valuable feasible new tool for parasitic vaccine production.

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التوكسوبلازما الخلقية: دور اللقاح المجهز بالميكروويف في منع انتقال العدوى من أمهات فئران التجارب إلى الأجنة

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المستخلص. درس البحث فعالية تحصين أربعة أنواع مختلفة من لقاح التوكسوبلازما المجهز بالميكروويف في حماية أمهات فئران التجارب وأجنتها وقدم دراسة طفيلية وباثولوجية. قسمت إناث الفئران إلى أربع مجموعات وتم تحصينهم قبل التزاوج. المجموعة الأولى حصنت بلقاح التوكسوبلازما المعد بالميكروويف والمحضر من الطور الخضرى والمجموعة الثانية حصنت بلقاح التوكسوبلازما المحضر من الطور الخضرى بالإضافة إلى الانترلوكين ١٢. أما المجموعة الثالثة حصنت بلقاح التوكسوبلازما المحضر من الطور المتحوصل والمجموعة الرابعة حصنت بلقاح المحضر من الطور المتحوصل بالإضافة إلى سم الكوليرا. تم تعرض جميع المجموعات بين اليوم ١٠-١٤ من الحمل لطفيل التوكسوبلازما (RH). أظهرت النتائج أن التطعيم المحضر من الطور المتحوصل وفر أكبر حماية للفئران عندما تم إضافة سم الكوليرا إليه كعامل مساعد تلاه اللقاح أو التطعيم المحضر من الطور الخضرى الذي أضيف له الانترلوكين ١٢. والجدير بالذكر أن إعطاء الانترلوكين ١٢ وكذلك سم الكوليرا كعوامل مساعدة مع اللقاحات أدى إلى زيادة قوة تأثير اللقاحات في

حمايـة الفئران وظهـر ذلـك مـن الدراسـة الباثولوجيـة حيـث إن فحـص أعضـاء الأمهـات مـن الفئـران التـي تـم تحصـينها باللقـاح المعـد مـن حويصلات التوكسوبلازما بالإضافة إلى سم الكوليرا كانت قريبة الشبه بأعضاء الفئران الطبيعية وكذلك هو الأمر بالنسبة لأعضـاء الأجنـة. وعلـى ذلك من الممكن الاستفادة من هذة اللقاحـات لـيس فـي الفئران فقط بل مع الثدييات التى تصاب بالتوكسوبلازما بما فيها السيدات.