The Effect of the Natural Milk Component, Lactoferrin, in Protecting Neonate Mice from Congenital Toxoplasmosis: An Experimental Study

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Abstract: Background: Lactoferrin (LF) is a natural cheap and easily prepared milk component with multifunctional iron glycoprotein fraction. It is well known to exert a broad spectrum antimicrobial activity against many bacteria, virus, yeasts and protozoa. Aim of the work: To evaluate the efficacies of LF alone or in combination with irradiated Toxoplasma vaccine for their ability to elicit protection in Swiss pregnant mice and their pups against challenge infection with Toxoplasma gondii. Material and methods: Pregnant mice were preimmunized before mating either by LF alone, irradiated Toxoplasma vaccine alone or combination of both preparations. Dams were then challenged by virulent T.gondii between 10 and 14th day of gestation. Results: The preliminary findings showed that preimmunization with the natural milk component, LF, before pregnancy was immunogenic and protective against T.gondi for both pregnant mice and their offspring as well. Moreover, the use of irradiated vaccine in combination with LF has proved to be more protective but with no significant differences. Conclusion and Recommendation: Since LF is effective, safe, natural and easily prepared from cow’s milk, supplementation as a food therefore could be used as a prophylactic dietary supplement without side effects specially for women during pregnancy.

Keywords: Toxoplasma, Congenital Toxoplasmosis, Lactoferrin, Ha’il, Saudi Arabia

1. Introduction

Toxoplasma gondii (T.gondii) is an obligatory intracellular parasite, affecting a wide range of population all over the world. The disease can be serious if acquired congenitally where it can cause severe damage to the nervous system of the fetus (Wong & Remington, 1994).

When a woman is infected for the first time during pregnancy, maternal immune response seems to protect the mother but not the fetus (Roberts et al, 1994). Previous studies have shown that cell immunity is depressed during pregnancy but recent studies have proved that rather than a depressed immune response, a predominant type 2 like response (Mouhate et al, 2008).

Immunization trials against acquired Toxoplasma infection are numerous and have been already documented mainly by vaccines (Araujo, 1994) (Jongert et al., 2009) (Debard et al., 1996). On the other hands, trial attempts for immunization against acquired Toxoplasmosis using immunostimulant like LF are very few (Shereen&Mossallam, 2009). Unfortunately studies in immunization against congenital toxoplasmosis are deficient and up to our knowledge, no studies were conducted to detect the protective role of immunostimulant or adjuvant fractions like LF against congenital toxoplasmosis. Lactoferrin is a natural cheap and easily prepared milk component with multifunctional iron glycoprotein fraction. It is well known to exert a broad spectrum antimicrobial activity which is probably due to that ability to sequester free iron required for microbial growth (Baker E.N., & Baker H.M., 2005).
was tried using in Mic1-3 Knockout of Toxoplasma gondii (Ismael et al., 2006) with significant reduction of vertical disease transmission.

Most of the candidate vaccines have been given parenterally in order to stimulate the systemic immunity which is known to be more effective in prevention of congenital toxoplasmosis. (Araujo et al., 1984 / Ismael et al., 2006). However in the view of natural protection, by T. gondii of the intestinal tract, induction of first line of local defense through oral immunization would also be of great interest.(Debard et al.,1996). Oral immunization by live vaccines against T. gondii infection has been tried to induce immunity against vertical transmission of in mice with subsequent good protection (Remington et al.,1961; Robert & Alexander, 1992). However the use of such cysts after killing by irradiation or the possible combination with the immunostimulant LF for protection of offspring against congenital infection has not been investigated previously.

2. Aim of the Study

Therefore, The main objective of the present work was to detect the possible immunization capacity of oral immunization with the natural component of milk LF ;irradiated Toxoplasma vaccine . In addition, the role of combination of such vaccine with LF as an immunostimulant was also tried to convey immunity to the newborns of the experimentally infected pregnant mice.

3. Material and Methods

1) Mice
Female Swiss albino mice were mentained and bred in the laboratory.

2) Parasite
a) RH strain of T. gondii was mentained by serial intraperitoneal passages of tachyzoites in mice. It was used for challenge of the pregnant dams.

b) KSU strain of T. gondii was mentained by serial L.P passage of the ground brain tissue into mice. It was used for production of the vaccine.

3) Vaccine Production:
Irradiated cyst vaccine (ICV); Gamma rays (cobalt 60) were used as radiation source and applied at a dose of 0.6 kGy (Song et al, 1993). Infectivity of the cysts was tested by bioassay of the radiated homogenate into Swiss mice. An amount of the homogenate, containing 5-10 irradiated cysts, was considered the immunized dose of the IVC and it was given orally just after being pregnant (Song et al., 1993 & Dubey et al., 1994).

4) Bovine Lactoferrin:
Iron saturated bovine LF (bLF; Sigma, Bornem, Belgium), with 90% purity (SDS-PAGE) and > 85% iron saturation purified from bovine colostrum, was used in this study in a dose (0.5 mg /kg / day). LF was daily administered to each mouse by a feeding syringe. The inoculation began one week before mating and then was continued till the end of the experiment (Bhimani et al., 1999)

5) Grouping schedule of mic:
Pregnant mice were divided as follows:

a) Control groups:
1-Normal control group(15 mice): Normal age matched female virgin mice were allowed to mate with males for one week during which females were examined for the presence of vaginal plugs and sperms in the vaginal fluids .The gestational day (zero- day ) was considered as soon as the sperms were found in the vaginal lavages.

2-Non-immunized-Infected control group(15 mice):
They were housed with males as the normal control mice. After mating, they were infected on the 10-14th day of gestation by subcutaneous (S.C) injection of 200 tachyzoites of the RH- strain/mouse, which is the minimal dose so that the mother mice could be able to deliver and rear their youngs successfully despite of being infected (Johnson, 1994).

b) Experimental group (immunize-infected):
They were further subdivided into the following subgroups:
- Subgroup S1: Mice received ICV alone (15 mice).
- Subgroup S2: Mice received ICV-LF combination (15 mice).
- Subgroup S3: Mice received LF alone (15 mice). After immunization, mice of the four subgroups were housed with males for mating . They were then challenged with the same dose and the same duration of the infected control mice.

3-Immunized-non infected control group: These mice were categorizes as follows:
- Mice immunized with ICV alone (15 mice)
- Mice immunized with ICV-LF combination (15 mice)
- Mice immunized with LF alone (15 mice)

** Immediately after the last dose of immunization ,they were allowed to mate normally.

6-Assessment of the effect of the LF and vaccine:
Pregnant dams of all groups were killed near full term (18-20 days) to prevent mothers from eating the pups with congenital anomalies if they were delivered in normal way. Blood samples were collected to assure infection (in the infected groups) using modified double sandwich ELISA (Turner ,1983) . Pups were removed intact with their placenta inside their uteri (fig 1 a) by caesarian sections

**Assessment of the effect of vaccine LF was done through the following:

a. For the offspring
1)Mean number of pups /each litter (full term uterus)
2)Total number of living pups /group.
3) Number of pups with congenital anomalies/group.
4) Determination of pups with congenital infection was done through Giemsa stained cord or placental blood films

*** Just after birth, Blood films from cord blood of pups were initially examined. Pups with positive parasitaemia were considered infected.
b. For Dams
1) Number of littering mothers (reaching full term) /group.
2) Number of aborted cases/ group.
3) Number of mothers died before littering/ group.
4) Number of mothers with abnormal placentae and uteri / group.

Statistical analysis
It was done by using X2 and Z test. 5% level was judged as a level of significance (Knapp,& Miller, M.C., 1992).

4. Results

Table I: Vertical disease transmission of the offspring born from the vaccinated mothers before receiving challenge infection with the virulent RH- strain of T. gondii

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal control gr.</th>
<th>Infected Control gr.</th>
<th>Subgroup S1</th>
<th>Subgroup S2</th>
<th>Subgroup S3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.of pregnant dams</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Total No.of pups</td>
<td>122</td>
<td>29</td>
<td>68</td>
<td>102</td>
<td>94</td>
</tr>
<tr>
<td>Mean No.of pups/litter</td>
<td>8</td>
<td>2</td>
<td>6</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Total No. of living pups</td>
<td>120</td>
<td>12</td>
<td>50</td>
<td>92</td>
<td>85</td>
</tr>
<tr>
<td>Survival rate (%)</td>
<td>98.3</td>
<td>41.4</td>
<td>73.5*</td>
<td>90.1**</td>
<td>80.4**</td>
</tr>
<tr>
<td>Total No. of dead pups</td>
<td>2</td>
<td>7</td>
<td>18</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Death rate (%)</td>
<td>1.7</td>
<td>58.6</td>
<td>26.5*</td>
<td>9.9**</td>
<td>19.6**</td>
</tr>
<tr>
<td>No of pups with cong. Anomalies</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rate of con g. Anom. (%)</td>
<td>0</td>
<td>34.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No. of infected pup</td>
<td>0</td>
<td>27</td>
<td>12</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Rate of VDT (%)</td>
<td>0</td>
<td>93.1</td>
<td>17.6*</td>
<td>9.8**</td>
<td>6.4**</td>
</tr>
</tbody>
</table>

*: Significant (P<0.05).
**: Highly significant (P<0.01).

Table II showed that frequency of littering was (40%) in the infected control mothers .The experimental subgroup mothers had higher levels of littering (66.7, 86.7, 86.7%) in S1, S2 & S3 respectively.
Out of the 15 control infected pregnant mothers, 6 mothers (40%) ended in abortion while the abortion rate decreased in the 3 subgroups.

At the end of the experiment, the calculated death rate was (20%) in the infected control mothers, and (13.3%) in those of subgroup S1 that received ICV alone. No deaths could be detected in those mice of subgroup S2 or S3 respectively. All uteri and placenta of the experimental subgroups were completely healthy except for subgroup S1 that received ICV alone in which 26.7% of their uteri and placenta showed similar pathological changes.

The immunized - non infected control group mothers and their pups showed similar results to those of the normal control pregnant dams and their pups as well.

Figure 1:

- a) Normal mother showing normal uterus
- b) Normal healthy pup
- c) Normal healthy placenta
- d) Normal healthy full term uterus
- e) Toxoplasma tachyzoites in placental smear
- f) Pup with congenital anomaly (vesiculation)
- g) Pup with congenital anomaly (maceration)
- h) Abnormal placenta with atrophy and necrosis
- i) Abnormal uterus with inflammation and gangrene

5. Discussion

The development of prophylactic means capable of inducing both local and systemic immunity is a valuable approach for use against the invasive infectious microorganisms such as *T. gondii*. Mucosal rather than parenteral rout of delivery of the vaccine candidates has been found to be the most relevant vaccine strategy to achieve these dual goals (Debard et al., 1996). To fulfill these goals we have investigated the possibility of use of oral irradiated cysts of *T. gondii* to induce protection against congenital toxoplasmosis. In addition, LF as a well known immunostimulant, has been tried alone or in combination with an irradiated toxoplasma vaccine to enhance the protective effect of the cyst vaccines.

The oral rout of administration was chosen on the basis that the natural entry of *T. gondii* through the oral intake. So, the protective immune response at the mucosal level is required to kill the parasite as soon as it penetrate the intestinal barrier. The probable major roles played by both CD8 T cells and antibodies specially IgA suggests that the best strategy would be to stimulate both cellular and humoral arms of the mucosal immune system. In addition, mucosal denderitic cells have been shown to induce good protection against both oral and parental *Toxoplasma* challenge (Debard et al., 1996) (Miller et al., 2009) (Buzoni et al., 2006) (Dimier-Poisson et al., 2003). So Our hypothesis was that an acceptable and effective vaccine would have to carry the optimized vaccine with or without an appropriate immunostimulant to target the mucosal denderitic cells by being endocytosed by the cells of the gut associated lymphoid tissues.

Challenge of the pregnant mice in the present study was performed by the use of the virulent Rh-strain of *T. gondii* on the 10-14 day of gestation. This time was found to be the most optimum time at which the pregnant dams give birth to infected offspring (Lee et al., 1983). We have also confirmed these results and our additional observation indicated that infection earlier during pregnancy ended in resorption or abortion of pups.

Numerous vaccination trials have been performed for protection against acquired toxoplasmosis. However, little is known about the use of such vaccination for conferring resistance against congenital toxoplasmosis.

The results of the present study demonstrated that pre-treatment of pregnant mice with the irradiated *T. gondii* cyst vaccine resulted in protection of both dams and their pups against challenge infection with the Rh-strain of *T. gondii*. The highest level of protection was noticed in the dams received the combination of the vaccine and LF (S2) followed by those mice received the irradiated vaccine (S1). The mechanism of resistance to the obligate intracellular organism *T. gondii*, is mainly cellular response. Cellular rather than humoral response appears to be partially dependant upon the sustained release of antigenic stimulus provided by the vaccine. Experiments comparing the protective efficacy of living and killed vaccines against intracellular parasites have usually shown the living vaccines to be far superior (Buxton et al., 1991) (Robert et al., 1994). However Live vaccines have a lot of side effects. So killed vaccine was chosen to immunize mice in the present study.

The killed vaccine (ICV), when used in the current study, was found to elicit less protection than use of LF alone or vaccine –LF combination for both mother dams and their pups. Few works have reported few success with killed *T. gondii* vaccines specially with the formaline killed vaccines and other workers failed. (Innes E.A, Vermeulen., 2006) (Waldeland & Frenkel., 1983) They explained their weak results on the basis that the killed antigen preparation, when administered alone, only elicits humoral immunity and rarely induce a state of delayed hypersensitivity which is considered an important recognition phase of cell mediated immunity. They also added that the combination of the antigen with adjuvant often does induces a state of delayed hypersensitivity. Thus a dual humoral and cellular response resulting from immunization with the ICV-LF combination in the current study, is a possible explanation for the greater level of resistance seen in this group as compared to that.
The mechanisms by which LF can act against Toxoplasma gondii parasites were found to be various. Omata et al., 2001 found that the infectivity of Toxoplasma gondiiis reduced after their incubation with LF and proved that LF breaches parasitic membrane integrity causing subsequent changes in interactions between the host and the parasite (Omata et al., 2001).

Furthermore it was found that the competition for iron between the parasite and LF is the basis of its ant-parasitic activity against protozoal infection(Cirioni et al., 2000). (Orsi et al., 2004) (Estrada et al., 2012) Another mechanism was that natural oral milk component could evoke immunity of the mucosal system of the intestinal tract with priming of the immune system at a more distant site which are the systemic immune sites (Shereen & Mossallam, 2009).

Many celltypes, including human lymphocytes and intestinal epithelial cells from young adult mice were proved to bear Lf receptors (Suzuki &lonnerdal b, 2002) .Moreover, the detection of bovine LF binding receptors on many bacteria and parasites has also been proved (Suzuki et al., 2005)

LF was successfully used to treat many bacteria likeLegionella,Stapaureus, Escherichia coli and Listeria monocytogenes(Zimecki et al., 2004) (Byrd, Horwitz,. 1991) (Bhimani et al., 1999) (Ice et al., 2004) .The antifungal activity of Lf has been mainly studied towards Candida with potential usefulness. (Viejo et al., 2004) As to the antiviral activity of LF, it is exerted against several viral infection like rotavirus,respiratory cyncitial virus and Herpes virus (Beljaaret al. 2004). ( Shimizu et al., 1996) (Fujihara& Hayashi,.1995)

The powerful augmentation as well as the successful protection by the exogenously supplied LF might be contributed to the state of immunomodulation. Thus it could overcome the state of immunosuppression normally present during pregnancy which is known to be due to the predominant type II immune response (Shereen&Mossallam, 2009) . Another explanation is that LF might reverse the associated impairment of the individual NK cells which are the main factors responsible for immunosuppression in pregnancy (Kuhara et al., 2006) .Although non specific immune response may have been a beneficial third component of resistance in the LF injected mice, it is more likely that activated macrophages would have an important role (Yamauchi et al.,1998).

Detection of the tachyzoites in the cord blood alone is a sure indication of infection. It is considered likely that parasitaemia represents the presence of infective leukocytes rather than free organisms within the blood (Roberts &Alexander ,1992). The mechanism by which the parasite transfers through the placenta may be wandering of infected leukocytes through cyncitialtrophoblast into the fetal circulation or due to ruptured of the parasitized white cells near the placenta to allow liberated organisms to make their way across the membrane.

The presence of the parasites in the peripheral blood in the current study may be due to transmission of the parasites either directly by blood containing parasites or because of the presence of foci of the parasites in placental tissue as well. (Fux et al., 2000). Application of the results of the current study on human specially during pregnancy, might be of great value as many published studies that haveexamined the use of Lf as a food supplement and itseffects on immunity have been quite promising (Weinberg., 2003) (Mulder et al.,2008) (Ishikado et al.,
2010). An interesting study using healthy human volunteers found that taking orally commercially available capsules containing bovine LF, derived from bovine milk, for 10 days indicated that LF may be applied in the clinics and hospitals to improve the immune status of the patients whether they are normal or immunosuppressed (Zimecki et al. 2008).

6. Conclusion

The results of the present work demonstrated that preimmunization with the natural milk component, LF, before and during pregnancy was immunogenic and protective against T. gondii for both pregnant mice and their offspring as well. Moreover, the use of irradiated vaccine in combination with LF has proved to be the most powerful protective preparation.

7. Recommendations

1) Since LF was safe, natural and easily prepared from cow’s milk, supplementation as a food therefore could be used as a prophylactic dietary safe supplement without side effects specially for women during pregnancy.

2) We would like to conduct a clinical trial on volunteer pregnant women to evaluate the use of LF alone in protection against Toxoplasma gondii infection in the future.

3) Further studies are warranted to determine if the use of LCV-LF combination is safe during pregnancy so that we would have a handful vaccine for human.

References


Author Profile

Prof. Safia Moussa received her MBCh (Bachelor Medicine) 1986, master degree (1993) and doctorate degree (1996) in Microbiology and parasitology all from faculty of Medicine

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