Effect of Levamisole and Vitamine E/ Selenium on Bovine Cellular and Humeral Immunity after Bovine Viral Diarrhea Vaccination

Abstract
The aim of the present study was to determine the effect of levamisol and vitamin E and selenium on bovine immunity associated with Bovine viral diarrhea (BVD) vaccination. Fifteen mixed breed calves at (Age, 5-8 months) of age in a private farm were used. Experimental animals were allocated randomly into 5 groups (3 each). Group 1 injected by Pneumo-3 vaccine (double doses of 5ml i.m with two weeks interval). Group 2 received Pneumo-3 vaccine and levamisole subcutaneously at a dose rate of 2mg/kg b.w for 2 successive times (3 days apart). Meanwhile, Group 3 received Pneumo-3 vaccine plus vitamin E/Selenium at a dose rate of 2ml/40 kg b.w, i.m, for 3 successive times (seven days interval). For group 4, Pneumo-3 vaccine plus levamisole and vitamin E/selenium (as previously mentioned) were administered. However, group 5 received only levamisole and vitamin E/selenium combination. Host humoral and cellular immune responses were evaluated before vaccination and at 2, 4, 8, 12, 16, 20, and 24 weeks post-vaccination. A significant effect of time and treatment (ANOVA P < 0.01) were recorded. The antibody titer against BVD virus in vaccinated calves reached its peak level at 8 weeks from first injection. The highest level of antibodies against BVD virus was observed in calves administered Pneumo-3 plus levamisole Hcl plus vitamin E and selenium. However, the apoptotic lymphocytes index and Acridine orange stain (AO) was parallel; the apoptosis was found to be the highest in second group and the lowest in third group. Agarose gel electrophoresis for DNA of apoptotic lymphocytes showed fragmentation of DNA. The results of the present study indicate that using of Pneumo-3 vaccine together with vitamin E and levamisol could induce a good humoral and cellular response to BVD in calves.

Keywords: Levamisol Hcl; Bovine; Cellular and humoral immunity; Vaccination; Vitamine E/ Selenium

Abbreviations: AO: Acridine Orange Stain; BVD: Bovine Viral Diarrhea; BVDV: Bovine Viral Diarrhea Virus; NCP-BVDV: Non Cytopathic-Bovine Viral Diarrhea Virus; GSH: Glutathione; TCID₅₀: Tissue Culture Infected Dose 50; dpi: Day Post Infection; MDBK: Madin Darby Bovine Kidney; HBSS: Hank's Balanced Salt Solution; SNNT: Serum Neutralization Test; PBS: Phosphate Buffer Saline; HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); NS3: Non-Structural Protein 3; PBLs: Peripheral Blood Lymphyocytes

Introduction
Bovine viral diarrhea is one of the production limiting diseases in dairy herd as it interferes with reproductive and immunological functions [1]. BVDV can affect the cell mediated immunity in buffalo with noticeable effect on humoral mechanism [2]. The success of vaccination aimed at increasing immunity and reducing the risk of infection. The transmission is likely depending up on the homogeneity between the vaccine strains and the field strains present in the herd. The more homologous the strains, the more cross- protection will be achieved. The goal of immunization is to stimulate both the B- and T- cell arms of the immune system. The B-cell has the major responsibility for inactivating free virus that achieved primarily by immunoglobulin, which neutralizes the BVDV infectivity and secondarily aggregated BVDV and enhances clearance. Cell- mediated immunity, particularly CD₄⁺ cells type 2, which is important for the resolution of acute infection with non-cytopathogenic BVDV (NCP BVDV) [3].

Selenium and Vitamin E independently enhance the immune response of lambs challenged with a viral pathogen. Selenium and vitamin E deficiency could compromise the immune system and result in a decline in resistance of animals to infections [4].

Levamisole seems to potentiate 5-fluorouracil’s antiproliferative effect in different tumor cell lines possibly by modulation of phosphorylation processes relevant for both cell cycle progression and apoptosis and this also for endothelial cells [5]. Levamisole induces apoptosis and growth arrest in cultured vascular endothelium to be associated with a loss of survival and antioxidative factors as well as with induction of growth arrest/ death signals [6]. Such Levamisol induces apoptosis seems to relate to oxidative stress which is counteracted by the antioxidants GSH and N-Ac and thus possibly depends on exhausting of GSH and/or a concomitant increase of intracellular H₂O₂ [7].

To the best of our knowledge, scare studies are available on use of levamisole and vitamins E for modulate the immune response associated with BVD vaccination. Therefore, the objective of the present study was to assess effect of levamisol and vitamin E/
selenium on bovine cellular and humoral immunity after bovine viral diarrhea vaccination.

Materials and Methods

Animals

A total of fifteen clinically normal calves (age, 5-8 months) at a private farm in Dakahlia Governorates, Egypt were used. Calves were randomly selected and allocated into five groups (3 each). Calves were fed on concentrated ration. Animals were not vaccinated for BVD.

Vaccine

A combined inactivated respiratory virus’s vaccine (pneumo-3, Veterinary Serum and Vaccine Research Institute, Cairo, Egypt) was used. The vaccine is used for protection against BVD, IBD, and PI3.

Cell culture

Madin Darby Bovine kidney (MDBK) cell line was used for both viral isolation and SNT [8]. The cell line was proved to be free from non-cytopathic BVD virus.

Virus strains

BVDV genotype-1 and BVDV genotype-2 strains were used. A local strain of BVDV genotype-1 cytopathic strain (Iman-strain, Veterinary Serum and Vaccine Research Institute, Cairo, Egypt) was used for serological tests. The TCID₅₀ per 100 μl = 10⁷ at 14 days post infection (dpi) [9]. BVDV genotype-2 is strain 125 of BVDV genotype-2 cytopathic strains (Ames Iowa Laboratories, USA). The TCID₅₀ per 100 μl = 10³ at 14 dpi. For indirect fluorescent antibody technique BVDV antiserum (Veterinary Serum and Vaccine Research Institute, Cairo, Egypt) was used.

Experimental vaccination

Experimental animals were allocated randomly into 5 groups (3 each). Group 1 injected by Pneumo-3 vaccine (double doses of 5ml i.m with two weeks interval). Group 2 received Pneumo-3 vaccine (as previously mentioned) and levamisole (Ucimisole®, Norbrook Laboratories, UK) at a dose rate of 2ml/40 kg b.w for 2 successive times, 3 days apart. Meanwhile, Group 3 received Pneumo-3 vaccine plus vitamin E/Selenium (Vitesel®, Norbrook Laboratories, UK) at a dose rate of 2ml/40 kg b.w, i.m. for 3 successive times (seven days interval). For group 4, Pneumo-3 vaccine plus levamisole and vitamin E/selenium (as previously mentioned) were administered. However, group 5 received only levamisole and vitamin E/selenium combination.

Blood Samples

Two blood samples were collected from each calf before vaccination and at 2, 4, 8, 12, 16, 20, and 24 weeks post-vaccination for serum. One of the blood samples was collected on EDTA for lymphocyte separation for estimation of cellular immunity, whereas the second sample was collected into tube without anticoagulant for obtaining serum.

Virus titration

Serial tenfold dilution of used viruses was prepared in cold HBSS. 0.1 ml of each dilution was inoculated into each of four tissue culture tubes. Culture tubes were incubated at 37°C for two hours. Then maintenance media was added. Tubes were incubated at 37°C; media and were changed every three days. Culture was observed microscopically for specific cytopathic effect for 2 weeks. Virus titer was calculated according to standard method [10]. Titer was expressed as TCID₅₀ per 0.1 ml of used viruses and was kept frozen till used for Serum Neutralization Test (SNT).

Serum Neutralization Test (SNT)

The test was performed using flat bottom sterile micro-plates according to Cerberey and Lee [11].

Lymphocyte separation: Cells well obtained from whole blood using lymphocyte-separation solution (Ficoll-Hypaque Pharmacia, Uppsala, Sweden) [12]. Lymphocytes were washed twice with sterile PBS pH 7.6. Cells were suspended at 10⁷/ml in RPMI 1640 (GIBCO) containing 25 mM glucose 20mM L-glutamine, 92 µg gentamicin 20 µg ml. (Walkersville MD) and 25 mM HEPES buffer (pH 7.3, Gibco). Cells were suspended in sterile PBS (PH 7.2). The cell suspension were divided into 4 aliquots to measure the apoptosis at zero time (base time), then 24, 48, and 72h, cells were incubated at (37°C in 5% carbon dioxide). After incubation, cells were centrifuged (200xg, 5min), suspended in ice-cold cold PBS and analyzed on a coulter counter. At time 0 and at subsequent times, cells were removed from culture and counted on a hemocytometer. Cell viability was determined by trypan blue dye exclusion test, in which one volume of trypan blue (0.4%, Gibco) was added to 5 volumes of cells at room temperature for 5 minutes.

Detection of apoptotic cells

At time zero and at subsequent times, cells were removed from each culture, fixed in methanol, harvested on slides stained with May Grunwald Giemsa for assessment of the percentage of cells showing morphology of apoptosis [13]. By using oil immersion light microscope, the apoptotic lymphocyte percentage at different times was calculated. To identify engulfed apoptotic cells, acridine orange stain (AO) was used. Briefly, one drop of cell suspension was added to one AO solution (10µg ml in PBS), mixed gently on a slide and immediately examined with an Olympus HB-2 microscope with fluorescence attachment [14].

Assessment of apoptotic index: For this analysis in each case, the mean number of monocytic cells per field was registered in 15 randomly chosen fields. The apoptotic bodies were then counted in greater number of fields and expressed as percentages monocytic cells per case [15].

DNA Fragmentation: Total DNA was extracted with 0.5 ml saturated phenol followed by chloroform and isoamyl alcohol (24:1) before centrifugation at 7000g, 4°C for 10 minutes. DNA in the supernatant was precipitated by adding 0.02 ml of 5 m NaCl and 1 ml of absolute ethanol. After centrifugation at 9500 rpm for 15 minutes, the pellets were air-dried prior to suspending in 0.1 ml TE buffer (10 mM Tris-Hcl, pH 8.0, 1mM EDTA). RNA was eliminated by Rnase digestion (0.1 mg ml Rnase, incubated at room temperature for 1 hour). The DNA was electrophoresed using 1.8% agarose gel and visualized by ethidium bromide staining [16].

Statistical Analysis

Statistical analysis was carried out using GMP, SAS commercial statistical software program. Repeated measures MANOVA (with repeated measures on treatment and time) were used to determine the main effect of dose and time. Wilks’ Lambda test
was selected to evaluate within group interactions and evidence of time group interactions. Where Wilks’ Lambda test indicated a statistically significant difference between groups, one way ANOVA with Tukey-Kramer HSD post-hoc multiple comparison tests was used to identify which group was statistically different from the rest. Differences between means at p < 0.05 were considered significant.

### Results

#### Humoral response

Antibody titer was affected significantly by time and treatment (P < 0.01). The antibody titer against BVD virus in vaccinated calves reached peak of its level at second month from first injection (Table 1). The highest level of antibody titer against BVD virus in vaccinated calves was observed in calves administered Pneumo-3 plus Levamisole Hcl plus vitamin E and selenium.

#### Table 1: BVDV antibodies titer detection by SNT in different vaccinated groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>0 time</th>
<th>2 weeks</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>12 weeks</th>
<th>16 weeks</th>
<th>20 weeks</th>
<th>24 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>1.1 ± 0.1 a</td>
<td>1.4 ± 0.17 a</td>
<td>1.6 ± 0.1 a</td>
<td>1.7 ± 0.17 a</td>
<td>2.0 ± 0.1 a</td>
<td>1.6 ± 0.17 a</td>
<td>1.1 ± 0.1 a</td>
<td>0.8 ± 0.17 a</td>
</tr>
<tr>
<td>Group 2</td>
<td>1.3 ± 0.1 a</td>
<td>1.5 ± 0.001 a</td>
<td>1.6 ± 0.1 a</td>
<td>1.8 ± 0.001 a</td>
<td>2.0 ± 0.1 a</td>
<td>2.1 ± 0.001 a</td>
<td>1.2 ± 0.001 a</td>
<td>0.8 ± 0.1 a</td>
</tr>
<tr>
<td>Group 3</td>
<td>1.3 ± 0.1 a</td>
<td>1.5 ± 0.30 a</td>
<td>1.0 ± 0.1 a</td>
<td>2.0 ± 0.17 a</td>
<td>2.2 ± 0.1 a</td>
<td>2.1 ± 0.30 a</td>
<td>1.3 ± 0.1 a</td>
<td>0.8 ± 0.1 a</td>
</tr>
<tr>
<td>Group 4</td>
<td>1.4 ± 0.1 a</td>
<td>1.6 ± 0.17 a</td>
<td>1.0 ± 0.1 a</td>
<td>2.0 ± 0.17 a</td>
<td>2.3 ± 0.1 a</td>
<td>2.3 ± 0.17 a</td>
<td>1.5 ± 0.001 a</td>
<td>0.7 ± 0.1 a</td>
</tr>
<tr>
<td>Group 5</td>
<td>0.4 ± 0.1 a</td>
<td>0.3 ± 0.001 b</td>
<td>0.4 ± 0.1 a</td>
<td>0.7 ± 0.17 b</td>
<td>0.8 ± 0.1 b</td>
<td>0.6 ± 0.001 c</td>
<td>0.8 ± 0.1 b</td>
<td>0.7 ± 0.1 b</td>
</tr>
</tbody>
</table>

The variables with different superscript letters at the same columns are significantly different at P< 0.05.

#### Cellular response

The apoptotic lymphocytes index detected by Giemsa and A.O are parallel. The apoptosis was found to be higher (p-value) in the group 2 and lower (p-value) in the group 3 but no significant difference between both group 1 and group 2 was found (Table 2 & Figure 1). Agarose gel electrophoresis for DNA of apoptotic lymphocytes showed fragmentation of DNA as showed in (Figure 2).

#### Table 2: The mean percentage of pBLs by Acridine orange stain (AO) in different vaccinated groups.

<table>
<thead>
<tr>
<th>Tested group</th>
<th>0 time</th>
<th>24 hrs</th>
<th>48 hrs</th>
<th>72 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>First group</td>
<td>16.4 ± 0.3</td>
<td>13.27 ± 0.4</td>
<td>10.53 ± 1.1</td>
<td>8.03 ± 0.4 a</td>
</tr>
<tr>
<td>Second group</td>
<td>15.20 ± 0.6 b</td>
<td>13.17 ± 0.6 b</td>
<td>10.27 ± 0.7</td>
<td>7.60 ± 0.1</td>
</tr>
<tr>
<td>Third group</td>
<td>15.30 ± 0.7 b</td>
<td>12.53 ± 0.3 b</td>
<td>10.03 ± 0.5</td>
<td>7.03 ± 0.2</td>
</tr>
<tr>
<td>Fourth group</td>
<td>15.40 ± 0.4 b</td>
<td>12.77 ± 0.2 b</td>
<td>9.4 ± 1.6 b</td>
<td>6.7 ± 0.2 c</td>
</tr>
<tr>
<td>Fifth group</td>
<td>17.83 ± 0.5 a</td>
<td>14.33 ± 0.6 a</td>
<td>12.30 ± 0.6 a</td>
<td>9.47 ± 0.5 a</td>
</tr>
</tbody>
</table>

The variables with different superscript letters at the same columns are significantly different at P< 0.05.
Discussion

The concept of control of BVD infection has changed since the first description of the disease. Early attempts to control the infection were focused on preventing economic losses on a herd basis by prophylactic vaccination. No attempts were made to eradicate BVD systematically on a regional or national basis [17]. Combined inactivated respiratory virus vaccines (pneumo-3 or pneu-4 vaccine) were evaluated to control BVD infection in cattle [18].

In the present vaccination trails, we used commercially available pneu-3 vaccine (combined inactivated respiratory virus vaccine containing BVD, IBR and PI3 viruses) with Levamisole HCl and vitamin E and selenium as nonspecific immunostimulants to study active immunity in cattle calves. Serum neutralization test has been used for quantization of antibodies against BVDV. The test is sensitive, mostly specific and relatively simple to be performed [19]. The titers were expressed as the log10 of the inverse dilution, which protected 50% of the tubes as calculated by Reed and Munch [20] methods according to Mayer [10]. The antibody titer against BVD virus in vaccinated calves reach peak of its level at second month from first injection, where the highest level of antibody titer against BVD virus was observed in calves administered Pneumo-3 plus Levamisole HCl plus vitamin E and selenium. Our results are in agreement with the results obtained by Allam [21] who mentioned that SNT were increased at 28 days post initial vaccination and reach the highest level by the day 60 post initial vaccination. Antibody titer was estimated by SNT through collection of sera samples at 0 day, 2 weeks, 4 weeks, 8 weeks, 12 weeks, 16 weeks, 20 weeks and 24 weeks post vaccination according to Fulton et al. [22] who inoculated multiple groups of cattle at different times to detect the optimal time of vaccination by using of inactivated polyvalent vaccine containing BVD, IBR, PI-3 and BRSV. They vaccinated at 0 day and day 14.

Ghally et al. [23] compared between local combined inactivated respiratory viruses vaccine containing IBR, BVD and PI-3 (pneumo-3) and imported vaccine containing modified live PI-3, IBR viruses and inactivated BVD virus (cattle master 4). The authors reported that both vaccines provide a very good protective level against challenge.

On using the immunostimulants the higher titer of antibodies was observed in the group 4, which administered Pneumo-3 vaccine plus levamisole HCl plus vitamin E and selenium. Immunostimulants can be potentially used to stimulate the immune system and decrease the amount of antibiotics used for BVD. Vaccination stimulates the immune system to produce antibodies to specific pathogens. This method of immunomodulation has been in use for over 100 years, but doesn’t always produce the desired results. Non-specific immunostimulants can be used to enhance the immune responses prior to, following, or at vaccinations. They may also help the animal’s immune system overcome the immuno-suppressive effects of stress and exposure to infectious agents involved with BVD [24]. There are many drugs used to increase the resistance of the animal by improving the humeral and cellular immune response such as vitamin E [25] and selenium [26], where they appear to provide an efficient method of stimulating the immune system in a non-specific manner with few adverse side effects. Immunosuppressing compounds have the potential to counteract the effect of environmental or microbial immuno-suppressive factors. They may thus reduce morbidity and economic losses resulting from subclinical or mild infectious diseases [3].

The exact mechanism by which levamisole could enhance serum antibody response to infective agents is not known. It has been reported that levamisole enhances macrophage and T-lymphocyte function and reduces suppressor T-cell function. Because antibody formation to most infectious agents is T-lymphocyte dependent, the augmentation of the helper functions of these cells could enhance antibody production [27]. Levamisole hydrochloride effects on humoral and cell-mediated immune response in several diseases have been examined [28]. Levamisole described as antiproliferative and affect both adhesion and MHC class I molecule expression [29]. Endothelial cells exposed to levamisole exhibited considerable vacuolization and increased rates of apoptotic cell death as apparent by condensation of chromatin and the presence of apoptotic vesicles when compared to their individual control cells without added levamisole [2]. Levamisole selectively induces apoptosis in cultured vascular endothelial cells from different origins, but not accompanying fibroblasts. The observed induction of endothelial apoptosis by levamisole may explain its impact as an adjuvant in cancer treatment relating to vascular targeting [30].

Selenium is considered one of the most promising candidates for preventing cancer and when combined with vitamin E, they produced a synergistic effect on cell growth suppression due to the two agents working in tandem to cause further delay in cell cycle transit [31]. α-tocopherol succinate is the most commonly used form of vitamin E in vitro studies of cancer research. It is generally assumed that because α-tocopherol succinate is less hydrophobic than α-tocopherol, it is taken up more efficiently by cells [32].

Cytopathic biotype of BVDV was able to kill cells by apoptosis in PBMC cultures while the homologous non-cytopathic virus could not [33]. Cytopathic virus triggers apoptosis in monocytes by the expression of specific BVDV proteins rather than virus replication in infected cells. In this regard, the viral protein NS3 is likely candidate because it is a non-structural protein restricted to cytopathic viruses [34]. The fact that NS3 has proteinase activities in intriguing, since apoptosis may be triggered by certain proteinases in variety of cell types [35]. The apoptotic lymphocytes index detected by Giemsa and A.O are parallel. The apoptosis was found to be higher in the second group and lower in the third group but no significant difference between either first group or second group. Agarose gel electrophoresis for DNA of apoptotic lymphocytes showed fragmentation of DNA. These results were also recorded by Stern et al. [36] and McDonald et al. [37] who stated that apoptotic cell recognition and removal by phagocytes is critical for the restoration and/or maintenance of normal tissue structure and function. Macrophages engulf apoptotic cells before they lyse, thus preventing release into the tissue of potentially toxic and immunogenic intracellular substances. In addition, the binding and or uptake of apoptotic cells not only fail to induce macrophage secretion of inflammatory mediators, but actually inhibit their proinflammatory cytokine production following stimulation.

Conclusion

The results of this study highlight the importance of using...
Pneumo-3 vaccine together with vitamin E and Levamisole as non-specific immunostimulant to induce good humoral and cellular response to improve the calves’ resistance to BVD.

References


