Effect of mineral supplementation on humoral immunity against rabies vaccine in dog pups

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ABSTRACT
The present study was aimed to determine the effect of a specially formulated mineral supplement in shaping the humoral immune response of dog pups in response to anti-rabies vaccination. Mineral supplement was formulated based on plasma analyzed for macro and micro minerals randomly collected from 226 dogs. Twelve mongrel/non-descript dog pups (3 – 6 months) randomly allocated into two groups (n=6) were tested for stimulation of humoral immune system by assessing the antibody titre against rabies vaccine using Rapid Fluorescent Focus Inhibition Test (RFFIT) and in vivo Mouse Neutralization Test (MNT) for antibody detection post supplementation of formulated mineral supplement upto 28 days. Early and higher antibody titre (>1:4096) was recorded on day seven itself in the group of dog pups fed with formulated mineral supplemented as compared to non-supplemented group. Present study indicated that mineral supplementation prior to the anti-rabies vaccination may elicit quick and high level of protective antibodies. The findings may also be important in the event of post bite immunization, in combination with this mineral supplementation for quick protective antibody response.

Key words: Dog pups, Humoral immunity, Mineral supplement, Rabies vaccine, Rapid fluorescent focus inhibition test.

INTRODUCTION
Rabies is one of the important viral zoonosis reported from more than 150 countries. It poses a serious threat to both human beings as well as animals and is fatal, once clinical signs develop. It affects most of warm blooded mammals including pets, wild animals and livestock species. Dogs are the primary host as well as one of the carriers of the rabies virus (Heymann 2015). Huge economic losses of about US$ 12.3 million was recorded in livestock sector due to rabies (Knobel et al. 2005), moreover on an average 61,000 humans die annually due to rabies worldwide (WHO 2013). Of these, 1/3rd burden of global rabies is shared only by India. Since dog bite is major mode of transmission of rabies infection to human beings and between animals in countries like India, proper anti-rabies vaccination is mainly and only precautionary tool for prevention of rabies in both. About 17.4 million Indians are exposed to rabies every year and post exposure prophylaxis costs around $25 million (Menzes, 2008).

Vaccines protect dogs through developing humoral immunity which depends upon several factors including nutritional status of the animal. It has been mentioned that, health education plays an important role for creating awareness in the community about importance of wound cleaning, proper vaccination schedule and rabies immunoglobulins in Class III degree exposure (Mahendra et al., 1999). He reported no anti rabies vaccination done in 64% of the positive cases for rabies and no wound toileting in 86.8% positive cases. Studies on importance of minerals necessary for optimal immune function in ruminants have been reported (Underwood and Suttle 1999) but meager information is available regarding impact of supplementation of minerals as a whole on immunity in pet animals. Balanced trace mineral supplementation will allow the animal’s immune system to respond with peak efficiency to minimize mortalities and complications to develop especially in post bite cases, which require a very fast protective immune response. Deficiency of these nutrients causes weakening of humoral and cell mediated immunities due to alteration in components of the immune system leading to reduced disease resistance power and increased susceptibility to diseases (Fraker and King 1998; Powell et al. 2000; Thurnham and Northrop-Clewes 2004).

Effects of infection on nutritional Supplementation with multivitamins or mineral mixture as a whole had been shown to improve immune function in aged mice and human beings (Chandra 1992; Bogden et al. 1994). Trace elements are having an important role in maintaining the activity of a number of enzymes directly involved in important defense processes ongoing in the animal body (Prasad 1998).

The present research was undertaken to study the effect of given formulated mineral supplement on vaccinal
immunity in dog pups using anti-rabies vaccine as a model. This model was chosen with a view so that concerted practices could be adapted during vaccination for enhancing immunity at an early stage in order to protect the pets, domestic animals, wild animals and human beings from this dreadful disease.

**MATERIALS AND METHODS**

**Formulation of mineral mixture:** Mineral supplement was formulated after estimating mineral levels in plasma of randomly selected 226 dog pups reported to Referral Veterinary Polyclinic (RVP), ICAR-Indian Veterinary Research Institute (Table 1). Plasma samples were digested for mineral estimation (Yatoo et al. 2013) and based on the readings mineral mixture was formulated with composition (Table 1).

**Experimental design:** Apparently healthy twelve non-descript dog pups aged between three to six months were selected for the present research work. After acclimatization pups were divided into two group having six animals each. Group 1 pups were maintained on basal vegetarian diet devoid of any mineral supplement, in contrast Group 2 pups were provided with formulated mineral supplement (as per Table 1) along with basal vegetarian diet. Mineral supplements (2.5 gm total dose) were fed orally to six experimental pups of group 2. The experiment had the prior approval from Institutional Animal Ethics Committee. Detailed schedule of vaccination and collection of serum samples is given in Table 2.

Serum samples were separated under aseptic condition in eppendorf tubes and kept at -20°C. Later all serum samples were decomplemented by keeping in water bath at 56°C for 30 minutes. Detection and quantification of anti-rabies antibodies in canine serum was done by Rapid Fluorescent Focus Inhibition Test (RFFIT) and in vivo Mouse Neutralization Test (MNT). The antibody titre was calculated for assessment of kinetics of antibody response.

**Propagation and quantification of virus:** Rabies virus (Pasteur Virus (PV) strain) available in Division of Biological Products, ICAR-Indian Veterinary Research Institute Izatnagar was used in the present study. A stock of PV strain with known titre was prepared after its propagation in BHK-21 clone 13 cells (Paldurai et al. 2014). Stock virus having 1.22x10^7 FFU/mL titre was used in the present study.

**Quantification of rabies virus specific antibody using RFFIT and MNT:** The humoral immune response was assessed by measuring the neutralizing antibody titre using RFFIT and MNT, induced after immunization of pups with rabies vaccine. Heat inactivated serum was serially diluted fourfold in Glassgow Minimum Essential Medium (GMEM) having 10% fetal calf serum and mixed with equal amount (32 µL) of virus (1.22x10^7 FFU/mL) in cell culture plate. Plate was incubated at 37°C for 1 hr for virus neutralization. After the virus neutralization, 32 µL of BHK–21 cells was added in all wells and incubated at 37°C for 24 hrs in humidified chamber at 5% CO2 tension. Cell monolayer was washed with PBS and fixed with 80% chilled acetone (80/20 in PBS) and stained with rabies anti–nucleocapsid FITC (Fluorescein isothiocyanate) conjugate (Patel et al. 2015). Antibody titre in both the groups was calculated and compared.

In MNT, a fixed quantity of known virus (Challenge virus standard strain) was mixed with a serially-4-fold dilution of homologous serum and incubated at 4°C overnight for virus neutralization. Swish albino mice (2-3 weeks age) weighing around 10-15 gm were inoculated with 30 μl from each virus with serum dilution through intracranial route. Mice were observed for 21 days and numbers of mice survived were noted.

**RESULTS AND DISCUSSION**

The difference between Group 1 and Group 2 antibody mounting accessed using RFFIT and MNT is depicted in Fig. 1 and Fig. 2, respectively.

In RFFIT, higher and quick antibody response was observed in mineral supplemented group on day 7 onwards (1:4096 and above) as compared to control. Accordingly, steady increase in antibody titre was also observed in mineral supplemented dogs but to a significantly lower level (1:8) as compared to control. However, on day 28 both the groups showed equal antibody response (>1:4096 and above).

In MNT, between both the groups, mice of mineral treated group showed significant reduction (20% vs 70%) in mortality on day 7 as compared to non-mineral treated puppies. The difference between Group 1 and Group 2 antibody mounting accessed using RFFIT and MNT is depicted in Fig. 1 and Fig. 2, respectively.

**Table 1:** Composition of mineral supplement fed during experimental period.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity (gm/kg mineral supplement)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dicalcium phosphate</td>
<td>450</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>200</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>60</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>100</td>
</tr>
<tr>
<td>Magnesium oxide</td>
<td>100</td>
</tr>
<tr>
<td>Ferric chloride</td>
<td>65</td>
</tr>
<tr>
<td>Copper sulphate</td>
<td>1.5</td>
</tr>
<tr>
<td>Zinc oxide</td>
<td>15</td>
</tr>
<tr>
<td>Manganese oxide</td>
<td>8</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**Table 2:** Immunization and blood collection schedule of dog pups for evaluation of vaccinal immunity.

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>Mineral supplement</th>
<th>Dose of Vaccine (Rabipur®)</th>
<th>Day of blood collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (n=6)</td>
<td>Control</td>
<td>No mineral supplementation</td>
<td>1 ml, I/M(&gt;0.5 IU)</td>
<td>0, 7, 14, 21, 28</td>
</tr>
<tr>
<td>Group 2 (n=6)</td>
<td>Tests</td>
<td>Formulated test mineral supplementation</td>
<td>1 ml, I/M(&gt;0.5 IU)</td>
<td>0, 7, 14, 21, 28</td>
</tr>
</tbody>
</table>

Note: All dogs were given booster dose on 21st days
Nutrients (including minerals) have been shown to have impact on immune competence. The focus of present study was to evaluate protective antibody titre against rabies vaccine after providing formulated mineral supplementation in dog pups. In pet animals, reports focusing about effect of combination of minerals in enhancing immunity against infections are inadequate. Most of the trials are done to investigate effect of mineral supplementation on production, immunity and reproduction in livestock and poultry.

In the present study, early and protective antibody response was observed on day 7 itself using RFFIT in mineral supplemented group as compared to group devoid of mineral supplement. This shows that mineral supplementation in the animal being vaccinated may be an important additive especially in post bite cases of rabies wherein an early antibody response is of utmost importance for the preventing the mortalities. Similarly, significantly greater (P<0.05) production of rabies specific antibodies at 2, 4 and 6 weeks post vaccination in group of dogs supplemented with antioxidant nutrients as compared to control group was recorded (Heaton et al. 2002). Proper functioning of immune system requires appropriate levels of macro as well as micro minerals to be included in the diet of animals. Impact of zinc deficiency on various systems of animal body (epidermal, gastrointestinal, nervous etc.) including immune system had been stated previously (Hambidge and Walravens 1982). It has also been concluded that zinc deficiency resulted in a decrease in blood lymphocyte population and atrophy of spleen and thymus, thus impairing immune system (Fraker and King 1998). Role of zinc in maintaining normal functioning of immune system, its role in specific innate cell types (monocytes/macrophages and natural killer cells) was now an established fact (Haase and Rink 2009; Maares and Haase 2016). Significant reduction in antibody production by spleen cells in copper deficient animals was also recorded (Koller et al. 1987).

Further, RFFIT results showed higher response in the antibody titre (>1:4096 and above) in both groups on day 28. This may be due to the anamnestic response after administration of booster dose of vaccine, where as in treatment group the booster effect was obtained in the primary immunization. Such a quick and high titre of antibody response may be very important especially in case of third degree bites, which is difficult to manage even with post bite immunization schedule, including the use of immunoglobulins.

Results of in vivo MNT revealed noteworthy differences between groups with lesser mortality percentage observed in group 2 (mineral treated dog serum). But no significant difference in mortality % within group 2 was noticed due to the protective early antibody response probably achieved on day 7 itself, whereas in group 1 (non treated dog serum) there was a decrease in mortality % from day 0 to 28.

CONCLUSION

Adequate balance of minerals in a pet’s body is extremely important in maintaining a healthy immune system thus in turn protects animals from dreadful diseases. While studying the relationship of minerals to health, it becomes increasingly evident that keeping a balanced level of minerals in every organ, tissue and cell of the animal body may be a prominent key to maintaining a healthy existence. The present study conclude with the findings that supplementation of minerals with special reference to trace minerals create a great impact on animal’s nutritional health in general. This supplementation may become important strategy for management of post bite cases due to early protection...
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conferred on account of quick immune response. Such strategies may be important for protection of domesticated animals subsequent to rabid dog bite cases, in the countries where rabies is endemic.

REFERENCES


