IMMUNOMODULATORY IMPACT OF METAMITRON IN BLACK BENGAL GOATS FOLLOWING REPEATED ORAL ADMINISTRATION

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ABSTRACT

The study was conducted to evaluate immunomodulatory effect of the herbicide, Metamitron in goats. Nine Black Bengal male adult goats were divided into three groups, each containing 3 animals. Control (Gr-I) group received carboxy methyl cellulose, while experimental control (Gr-II) group received levamisole at immunostimulating dose and experimental group (Gr-III) received Metamitron daily for 35 days. On day 36 all goats were immunized with 5% SRBC intraperitoneally. Animals were sacrificed for conducting Haemolytic Plaque Assay on day 5 post dosing SRBC. Total serum IgG level was estimated by Sandwich ELISA. No significant variation in serum IgG level was observed at different days against 0 day in control and experimental group. But serum IgG level was increased significantly in experimental control group. No significant variation was observed in percentage of plaque formation in control and experimental group but the value was significantly higher in experimental control group. Results indicated that Metamitron had no effect on immunomodulation of goats during this observation period.

Key words: Black Bengal goat, Haemolytic Plaque Assay, Immunomodulation, Sandwich ELISA.

INTRODUCTION

Immune system is an important indicator of toxicity assay of environmental contaminants and pollutants having residual effects in eco-system. Immunotoxicology explains clearly the adverse effects of environmental chemicals on immune system. These adverse effects are immunosuppression, immunostimulation, allergic or hypersensitivity reaction and autoimmunity (Luster et al., 1990). Livestock like cattle, goat, sheep etc, bio-accumulate pesticides due to improper application or persistence in the eco-system. Most of the pesticides are found to exert immunosuppression effect on both wings of immunosystem i.e. humoral and cell-mediated immunity. Immunosuppression was observed in goats fed with 41.6 ppm cypermethrin daily for 30 days (Tamang et al., 1988). Metamitron [4 amino 3 methyl 6 phenyl 1,2,4 triazine 5(4H) one] belongs to the family of triazine herbicide having probability and acceptability for field application to control weeds. Now a days considerable interest has arisen to study the immunomodulatory effect of agrochemicals. Study of immunomodulatory effect of Metamitron is scarely available. Considering the above, an attempt was made to evaluate the immunomodulatory effect of Metamitron.

MATERIALS AND METHODS

Clinically healthy nine Black Bengal male goats (1-1.5 year age) weighing between 9-12 Kg were caged individually in custom made metabolic cage (stainless steel).

Before starting the experiment animals were dewormed twice with a mixture of albendazole (7.5mg/Kg) and rafoxanide (7.5mg/Kg) at 21 days interval. After one month of last dosing of anthelmintic, animals were acclimatized for 7 days before starting the experiment. The present study was approved by CPCSEA (India).

Animals were divided into three equal groups. The experimental group (Gr-III) of goats were fed with Metamitron suspending in 10 ml of
1% carboxy methyl cellulose (CMC) at a maximum non toxic oral dose of 30 mg/Kg once daily for a consecutive period of 35 days, while levamisole was administered at standard immunostimulant dose 2.25 mg/Kg (Robertson, 1993) to experimental control (Gr-II) group of animals.

The Control group (Group-I) of goats received only the same volume of 1% CMC once daily for consecutive 35 days. On 36 day all goats were immunized with 5% SRBC intraperitoneally and on day 5 post dosing SRBC, animals were sacrificed for conducting Haemolytic Plaque Assay. Test chemical: Metamitron [4 amino 3 methyl 6 phenyl 1,2,4 triazine 5(4H) one] was provided by M/S Ghardha Chemicals Ltd., Mumbai. Purity of the compound was >98%. All chemicals and reagents used in this study were obtained from Sigma Chemicals Co. (USA) and Bangalore Genei(India).

Collection of samples: Blood samples were collected from all nine goats before administration of Metamitron (herbicide) (0day) and thereafter at every 7 days interval till 35 days post dosing. Serum was separated and preserved at -20°C for estimation of IgG level. A small portion of spleen from each goat was collected in ice-cold RPMI -1640 (pH-7.4) media for haemolytic plaque assay.

Estimation of serum immunoglobulin G (IgG) level: Before performing Sandwitch ELISA, goat IgG was purified by ion-exchange chromatography according to Himmelhcoch ,(1971). Anti goat hyperimmune sera was raised by repeated intramuscular injection of purified goat IgG in two rabbits. Anti- species conjugate and substrate were procured from commercial farm. Purification of goat IgG: Serum (15 ml) was collected from blood of a healthy goat and centrifuged at 3000 rpm for 15 min. Supernatant serum (12 ml) was collected and 8 ml saturated ammonium Sulphate (pH-7) solution was added slowly with constant stirring. After centrifugation at 3000 rpm for 30 min precipitate was collected and dissolved in 6 ml of distilled water. Precipitation was washed twice by addition of 4ml of distilled water. Final precipitation was dissolved in 4 ml of 0.15 M sodium chloride solution. The semi-purified protein was dialysed against 0.15M NaCl solution and redialysed against 0.04 M Phosphate buffer solution (pH -8) to make it equilibrium with the column. Before loading to column, protein concentration of the solution was measured by the method of Lowry et al(1951). A standard curve with known concentration (50 to 200 g) of bovine serum albumin was prepared earlier and used for protein estimation. The concentration of protein in the sample was 54.5mg/ml.

Isolation of goat IgG

Activation of DEAE-Cellulose: Diethyl amino ethyl cellulose (DEAE-Cellulose) was used as anion exchanger. DEAE-Cellulose (10 gm) was swelled in 250 ml of distilled water for 3 hr. Sediment was mixed with 250 ml 0.2 M Sodium hydroxide for 30 min and washed with distilled water until the supernatant became pH 7.0. Then the sediment was mixed with 250 ml of 0.2 M hydrochloric acid for 30 min and washed with distilled water until of the supernatant became 6.0. Again the sediment was washed few times with 0.01M Phosphate buffer (pH -7) until pH came to 7.0 and suspended in 50 ml of this buffer solution.

Packing of column: A glass column (60×2) was filled to about 10 cm with 0.01 M phosphate buffer (pH -7.0). Thick slurry of equilibrated DEAE-Cellulose was poured into the column until 5 cm gel was packed. No air bubble was entrapped. After proper packing the column was equilibrated with 0.04 M phosphate buffer (pH -8.0) until pH of the effluent buffer was 8.0.

Sample application and elution: 0.2 ml protein sample equilibrated with 0.04 M phosphate buffer (pH -8.0) was applied. phosphate buffer (0.04 M, pH -8.0)(elution buffer) was poured intermittently. Buffer 5 cm was always maintained above the top-level of packed DEAE-Cellulose. An elution rate of 16.5 to 17.0 ml/hr was maintained throughout the elution. Absorbance of the eluted material was collected at 3 ml fractions. Absorbance of eluted material of each fraction were measured by Spectrophotometer (Spectroscan UV 2600) at 280 nm wavelength. Tubes showing peak were pooled and the volume was concentrated by reverse osmosis using sucrose.

Sodium dodecyl sulphate –Poly acrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE (Laemmli,1970) was purified using 10% polyacrylamide resolving gel Protein (50 ml) was
loaded per lane and the gel was run for 30-40 min at 200V. The gel was stained by coomassie brilliant blue R-250 (0.125%W/V) in ethanol (40% V/V)-acetic acid (10%V/V) mixture and destined subsequently before studying the protein bands.

Production of anti-goat IgG hyperimmune serum (Anti-goat HIS): Anti goat HIS was raised by injecting goat IgG into the gluteal muscle of two rabbits. The procedure was as follows:

<table>
<thead>
<tr>
<th>Day</th>
<th>Amount of injected IgG (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>400</td>
</tr>
<tr>
<td>5</td>
<td>450</td>
</tr>
<tr>
<td>10</td>
<td>500</td>
</tr>
<tr>
<td>15</td>
<td>500</td>
</tr>
<tr>
<td>20</td>
<td>600</td>
</tr>
<tr>
<td>28</td>
<td>650</td>
</tr>
<tr>
<td>38</td>
<td>650</td>
</tr>
</tbody>
</table>

Agar gel precipitation test (AGPT): Agar (1%) was used for the test. Two fold dilutions of HIS were tested against undiluted goat IgG. After 4th injection, a titre of 1:8 or higher was obtained.

Collection of anti goat HIS: Samples were pooled and protein concentration was estimated by Lowry's method.

Sandwich ELISA test: Sandwich ELISA was performed by the method of Nemzek et al. (1999). Before this the appropriate dilutions of conjugate and coating anti-goat HIS were standardized by Chequer board titration.

Anti-goat IgG HIS was serially diluted with PBS (pH-7.4) and columns quadruple wells were coated with 100 l of each dilution. The plate was incubated overnight at 4°C. After washing with PBS (3 min for 4 times) wells were blocked with 5% (W/V) skim milk powder for 90 min at 37°C. Each well received 200 l. Further washing with PBS containing 0.1% (V/V) tween 20 (PBST) was done as before. The 0 day serum sample from one control goat was added (1:5000) dilution (100 l/well) and incubated at 37°C for 90 minutes. After washing (1:500, 1:1000, 1:2000, 1:4000) dilutions of conjugate were added (100 l/well) in duplicate rows. After incubation (37°C, 90 min) and washing 100 l of substrate solution was added in each well. Intensity of colour was read 450 nm after 10, 20 and 30 min of incubation at 37°C. Optimum anti-goat HIS dilution and conjugate dilution were calculated to be 1:8000 and 1:2000 respectively.

Antigoat IgG HIS 1:8000 dilution was used for coating (100 l/well). Following incubation, washing and blocking (100 l) of test serum sample (1:5000) (1:10000) were added to each triplicate well and incubated (37°C for 90 min.). After washing 100 l conjugate of 1:2000 Dilution was added to each well. 100 l of substrate was added to each well. After incubation at 37°C for 15 min the plate was read at 450 nm against blank (No HIS and HIS but no sample). IgG levels in serum sample were deduced from standard curve prepared before using fortified goat-IgG as capturing antibody.

Haemolytic plaque assay: Haemolytic plaque assay was done following the standard method of Jerne and Nordin (1963). Sheep blood (15 ml) with equal volume of Alsever's medium stored at 4°C for 8 days. After centrifugation at 1000 r.p.m for 10 min the SRBC in Alsever's Solution was washed with Phosphate Buffer Solution (pH-7.4) for three times. For immunization 5 ml suspension of 20% SRBC in PBS was injected intraperitoneally into all nine goats on day 36. Immunized goats were sacrificed on 5th day post immunization.

Preparation of petridishes with bottom layer: Agar [2.8% (W/V)] was prepared in distilled water and autoclaved. It was mixed with equal volume of prewarmed PBS (pH-7.4). This agar was poured into petridishes (60mm diameter).

Preparation of single cell suspension of immunized goat's spleen: Spleen was taken from immunized goats in 5-7 ml of ice-chilled RPMI-1640 Media. It was minced and suspension was filtered. Viable cells were counted by mixing equal volume of filtrate with trypan blue (0.1%) by trypan blue dye exclusion test (Hudson and Hay, 1980).

Preparation of SRBC for Top layer: The SRBC in Alsever's Solution which was used for immunization of goat was centrifuged at (1000 r.p.m for 10 min) washed thrice with PBS (pH-7.4). A 20% suspension (V/V) was made with PBS (pH - 7.4).

Preparation for top layer: Equal volume (0.5 ml) of agarose stock solution was mixed with prewarmed PBS (pH -7.4) to which 0.1 ml of 20% SRBC and 20% of 0.1 ml of spleen single cell suspension were added. Temperature of agarose was maintained at 45°C. After proper mixing this preparation was poured on bottom layer uniformly.

Incubation of petridish: After solidification of top layer petridish was incubated at 37°C for one hr. Complement (1.5ml) added to petridish by diluting
guineapig serum at 1:5 in PBS (pH 7.4). Then it was incubated for another 1 hr. It was poured from petridish. Plaques were counted with the help of magnifying glass.

**Statistical analysis:** The results were analysed using general linear model with invariable data in SPSS 10.0 version of software. The results were expressed as mean±standard error (S.E).

**RESULTS AND DISCUSSION**

**Serum IgG level:** Figure 1 shows clear separation of IgG and Fig 2 represents the standard curve of goat IgG. Serum IgG level at different days against 0 day of all three groups of animals is presented in Table-1. From Table 1 it is quite clear that the vehicle CMC had no effect on serum IgG level in control group of animal. The values of serum IgG level in experimental control (Group II) group of animals which received levamisole at 2.25 mg/Kg b.w.shows significantly (P< 0.01) increased serum IgG level. But serum IgG level was not changed throughout the period of observation in experimental group of animals (Group III) treated with Metamitron (30 mg/Kg) orally once daily for consecutive 35 days.

**TABLE 1:** Effect of Metamitron on serum immunoglobulin G (IgG) level (mg/ml) in Black Bengal male goat following successive oral administration for 35 days.

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
<th>35</th>
<th>41</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control I</td>
<td>13.25±0.30</td>
<td>12.5±0.40</td>
<td>10.75±0.24</td>
<td>12.25±0.22</td>
<td>13.0±0.26</td>
<td>13.25±0.07</td>
<td>11±0.30</td>
</tr>
<tr>
<td>'Experimental'</td>
<td>9.25±0.13</td>
<td>10.5±0.25</td>
<td>8.5±0.17</td>
<td>19.5±0.60</td>
<td>24.5±0.26</td>
<td>20.5±0.26</td>
<td>18.0±0.11</td>
</tr>
<tr>
<td>Control II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td>12.17±0.32</td>
<td>13.17±0.21</td>
<td>11.42±0.86</td>
<td>11.33±0.35</td>
<td>11.33±0.35</td>
<td>11.5±0.76</td>
<td>11.67±0.88</td>
</tr>
</tbody>
</table>

*Mean values with different superscript in a row are significantly different (P<0.01)*

1. Administered CMC (10 ml) once daily by oral route for consecutive 35 days.
2. Administered levamisole (2.25 mg/Kg) for consecutive 3 days followed by 3 days rest and again for subsequent 3 days by subcutaneous route.
3. Administered metamitron (30 mg/Kg) once daily by oral route for consecutive 35 days.

**FIG I:** Separation of goat IgG by Column Chromatography

**FIG II:** Standard curve of goat IgG.

**FIG III:** Agar gel precipitation test.
TABLE 2: Haemolytic Plaque Assay (%) by caprine spleen cell sacrificed on 5 day Post immunization with SRBC.

<table>
<thead>
<tr>
<th>Group</th>
<th>Percentage (%) of plaque formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.16 ± 0.12</td>
</tr>
<tr>
<td>Experimental control</td>
<td>20.00 ± 0.57</td>
</tr>
<tr>
<td>Experimental</td>
<td>7.07 ± 0.18</td>
</tr>
</tbody>
</table>

- Administered CMC (10 ml) once daily by oral route for consecutive 35 days
- Administered levamisole (2.25 mg/Kg) for consecutive 3 days followed by 3 days rest and again for subsequent 3 days by subcutaneous route.
- Administered metamitron (30 mg/Kg) once daily by oral route for consecutive 35 days.

Krakowski et al., (1999) observed the effect of levamisole and 1,3/1,6 glucan applied in pregnant mares parameters of non-specific cellular and humoral immunity of foals. The level of immunity was higher in foals from dams immunostimulated with levamisole was clearly exerted by this review.

Chronic exposure to DDT and another organochlorine insecticide which caused depressed immune response in rodents did not change vaccinal responses in exposed children (Descotes, 1999). From the above study it might be concluded that Metamitron, a triazine group of herbicide had no effect on immune status of goats during the 35 days observation period.

Haemolytic Plaque Assay: The percentage of haemolytic plaque by spleen cells in control (Gr-I), experimental control (Group-II) and experimental (Group-III) have been tabulated in Table-2. No significant variation in the percentage of plaque formation was observed between control and experimental group. But the value was significantly higher (P < 0.01) in experimental control group. Obminska and Calkosinski (1994) reported that levamisole potentiated the primary humoral response to SRBC by increasing the number of plaque forming cells and the level of anti-SRBC antibody. Significant variation in percentage of plaque formation in experimental control group compared to control group corroborated the above contention.

Estimation of plaques against SRBC in a heterologous host gives an indication of humoral immune status of the host. Direct IgM or indirect IgG plaques can be measured depending on the day of slaughter after immunization. In this experiment haemolytic plaque assay may indicate the presence of IgM as the animals were sacrificed on day 5 post immunization. The unchanged level of IgM by Metamitron indicated that it had no effect on humoral immune status of host during this observation period.

From the above study, it might be concluded that Metamitron had no effect on humoral and cell mediated immunity.

REFERENCES