IN VITRO EFFECT OF COW URINE DISTILLATE AND ITS SYNERGISTIC ACTION WITH AQUEOUS POLYHERBAL EXTRACT ON LYMPHOBLASTOGENESIS IN COMMERCIAL LAYER CHICKS

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ABSTRACT

The present investigation was undertaken to study *in vitro* effect of cow urine distillate and its synergistic action with aqueous polyherbal extract containing Ocimum sanctum (Tulsi), Tinospora cordifolia (Guduchi) and Withania somnifera (Ashwagandha) on lymphoblastogenesis in commercial layer chicks. A total of 270, day old BV-300 layer chicks were randomly divided into nine groups comprising 30 chicks in each group. Group I served as negative control without administration of any treatment except plain water *ad libitum*. Groups II and III served as positive treatment control groups and were administered with IBD intermediate vaccine and cyclophosphamide as immunosuppressive agents respectively. The remaining treatment groups IV to IX were given either cow urine distillate alone or in combination with aqueous polyherbal extract along with intermediate IBD vaccine and cyclophosphamide. Six birds from each experimental group were sacrificed on day 0, 15, 30, 45 and 60 for collection of materials for lymphoblastogenesis study. The assay revealed significant decrease ($P<0.05$) in T-cell blastogenesis in group III and B-cell blastogenesis in groups II and III as compared to group I on 30^{th} and 45^{th} day of experiment. There was significant increase ($P<0.05$) in T-cell and B-cell blastogenesis in groups IV and V as compared to group I, in groups VI and VII as compared to group II and in groups VIII and IX as compared to group III on 30^{th}, 45^{th} and 60^{th} day of experiment. These results suggested the potent humoral and cell mediated immune response of cow urine distillate and its combination with polyherbal extract in layer chicks.

KEYWORDS : Cow urine distillate, Lymphoblastogenesis, Polyherbal extract, Layer chicks

INTRODUCTION

Livestock and poultry population are affected by many infectious diseases which cause immunosuppression leading to failure of vaccination against these diseases (Ganguly and Prasad, 2011). In spite of timely vaccination by established methods, failure and breakdown of immunity has become common. Irrational use of antibiotics as growth promoter and as therapeutic agent or immunomodulator in livestock and poultry has also become a current issue. Day to day managemental practices imposes stress on chicks which leads to decreased performance and also reflects on immunity of chicks. Different managemental stress predisposes chicks to immunosuppression and exposes to infections. To overcome these immunosuppressive conditions, modulation of micro-environment of the immune system seems to be essential. This can be achieved by immunomodulators or immunostimulating compounds. Natural products of plant and animal origin offer a vast resource of newer medicinal agents with potential in clinical use **(**Ziauddin et al., 1996). Cow urine has been described in 'Sushrita Samhita' and 'Ashtanga Sangraha' to be the most effective substance/secretion of animal origin with innumerable therapeutic values. In Ayurveda cow urine is suggested for improving general health (Khanuja, 2007). It has also been found to be a very good immunoenhancer (Mishra et al., 2011).Many herbal plant preparations are prescribed to strengthen host resistance (Thatte and Dahanukar, 1989) due to their immunomodulatory activities.

One such plant, Tinospora cordifolia, commonly called 'Guduchi' has been examined for its immunomodulatory properties. Tulsi (Ocimum sanctum) has been reported to possess various medicinal properties like antibacterial, anti-inflammatory, immuno-modulatory and hepatoprotective (Gupta et al., 2002). Research reveals that ashwagandha *(Withinia somnifera)* possesses anti-inflammatory, antitumor, antistress, antioxidant, immunomodulatory, hemopoetic, and rejuvenating properties. There are reports available on immunomodulatory effect of cow urine distillate (Panicker et al., 2013 and Sharma and Jain, 2013) and herbal plant extract in laboratory animals. However, scientific data are not available regarding *in vitro* immunomodulatory effect of mixture of cow urine distillate and polyherbal extract in commercial layer chicken; therefore the present study was undertaken to study the effect of cow urine distillate and polyherbal preparation on lymphoblastogenesis in layer chicks.

MATERIALS AND METHODS

The present study was carried out at the Experimental Unit, Department of Veterinary Pathology College of Veterinary Science, AAU, Anand, Gujarat. For this study, cow urine distillate named "Surbhi Ark" was procured from Shri Aksharpursottam Swaminarayan Mandir Gaushala, Sarangpur which was made from fresh urine of young healthy Gir cows. The aqueous polyherbal extract used during the study was procured from Parnika herbal Pvt. Ltd., Surat. Intermediate plus infectious bursal disease vaccine prepared by Ventri Biological Limited, Pune and powder of cyclophosamide, Biochem Pharmaceutical Limited, Mumbai were used as known immunosuppressive agents for humoral immunity and cell mediated immunity respectively.

A total of 270 day old layer (BV-300) chicks were procured from Shakti Hatcheries Pvt. Ltd., Sarsa, Anand, Gujarat and were maintained under standard managemental conditions. The experimental procedures were reviewed and approved by the Institutional Animal Ethics Committee (IAEC). Environmental temperature and lighting regimes were applied according to the BV-300 guidelines. All the birds received a balanced layer pre-starter feed till the end of the experiment, in crumb form. Day old chicks were randomly divided into nine groups comprising 30 chicks in each group and numbered as group I to IX. Group I served as negative control without administration of any treatment except plain water *ad libitum*. Group II and group III served as positive treatment control groups by administration of IBD intermediate vaccine and cyclophosphamide respectively, as immunosuppressive agent. The remaining treatment groups IV to IX were given either cow urine distillate alone or in combination with aqueous polyherbal extract along with intermediate IBD vaccine and cyclophosphamide (Table-1). The birds were sacrified and lymphoid organs, thymus and bursa were collected aseptically from six birds of each group on day 15, 30, 45 and 60 and lymphocytes were isolated separately using media RPMI-1640.

Cell culture medium: One vial of tissue culture medium RPMI-1640 (HiMedia, India) was dissolved in one liter of triple glass distilled water and filtered through 0.22 μ membrane filter. The filtrate was distributed in aliquots of 100 ml and kept at 4°C until use. Before use, pH was adjusted to 7.2 ± 0.1 with the help of sterile 0.89 M sodium bicarbonate solution. Then after the medium was supplemented with 10 ml of sterile fetal calf serum (Sigma) containing 1000 IU of penicillin, 100 μg of streptomicin and 2000 IU of nystatin to check the contamination of fungus and bacteria in the culture medium.

Mitogen: Concanavalin-A (Con-A) at the concentration of 5 μg/ml and lipopoly-saccharide (LPS) at the concentration of 4 μg/ml was used as mitogens for T-cell and B-cell responses respectively.

Isolation of lymphocytes: Thymus and bursa collected from the experimental birds under aseptic conditions were cut into small pieces and suspended in media for separation of lymphocytes. Suspension was filtered through sterile muslin cloth and the cells were counted in filtrate using trypan blue (0.5%) dye exclusion test. Finally, the lymphocyte counts were adjusted as 10^6 cells / ml in

Table 1: Experimental Protocol

Group	No. of layer chicks	Treatment	Dosage and route of administration
I	30	Plain Water	*ad libitum* by oral route
II	30	Infectious bursal disease intermediate vaccine	On 13th day as recommended dose by eye drop
III	30	Cyclophosphamide	150 mg/kg b. wt. intravenous (i/v) once on 13th day
IV	30	Cow urine distillate	1 ml/kg b.wt. daily for 60 days by oral route
V	30	Cow urine distillate + Aqueous polyherbal extract	1ml/kg b.wt. + 2 ml/kg b.wt. } daily for 60 days by oral route
VI	30	Infectious bursal disease intermediate vaccine + Cow urine distillate	On 13th day of age as recommended dose by eye drop + 1 ml/kg of daily for 60 days by oral route
VII	30	Infectious bursal disease intermediate vaccine + Cow urine distillate + Aqueous polyherbal extract	On 13th day of age as recommended dose by eye drop + 1 ml/kg b. wt. + 2 ml/kg b. wt. } daily for 60 days by oral route
VIII	30	Cyclophosphamide + Cow urine distillate	150 mg/kg b.wt. intravenous (i/v) once on 13th day + 1 ml/kg of daily for 60 days by oral route
IX	30	Cyclophosphamide + Cow urine distillate + Aqueous polyherbal extract	150 mg/kg b. wt. intravenous (i/v) once on 13th day + 1 ml/kg b.wt. + 2 ml/kg b.wt. } daily for 60 days by oral route

RPMI-1640 medium and 2 ml suspension of lymphocytes each from thymus and bursa was prepared.

Test procedure: Lymphocyte proliferation assay was performed using Con-A as mitogen for T-cells and LPS for B-cells following the method described by Chauhan (1998). Triplicate cultures were made using 100 µl of cell suspension and 50 µl of medium alone or medium containing Con A/ LPS in flat bottom sterile micro titer plates (Corning, USA). Plates were sealed with cello tape and were incubated at 37°C in CO_2 chamber for 68 hrs. After incubation supernatant fluid from the wells was discarded and 50 µl 3-4-5 dimethyl thiazol (MTT) (5 mg/ml) (Sigma) was added to all wells followed by reincubation at 37°C in CO_2 chamber for 4 hr. 100 µl of acid isopropanol (0.04 N HCl in isopropanol) was then added to each well and the absorbance of each well was measured in

computerized micro scan ELISA reader at wave length of 570 nm. The values of triplicate wells were averaged and the mean optical density of mitogen stimulated cultures was obtained. The mean OD of control wells was subtracted from mean OD of wells with mitogen and presented as mean delta OD.

One way analysis of variance (ANOVA) and Duncan's New Multiple Range Test (DNMRT) were used to compare the effects of treatment of cow urine distillate and poly herbal extract on different variables in control and treated layer chicks by using software SPSS (Version 12.1).

RESULTS AND DISCUSSION

There was significant increase (P<0.05) in T-cell and B-cell lymphoblastogenesis in groups IV and V as compared to group I on 30th, 45th and 60th day of experiment. However, there was a significant (P<0.05) decrease in T-cell and B-cell lymphoblastogenesis in group III as compared to group I on 30th and 45th day of experiment. Additionally there was also decrease in B-cell lymphoblastogenesis in group II as compared to group I. There was significant increase (P<0.05) in T-cell and B-cell lymphoblastogenesisin groups VI and VII as compared to group II, while in groups VIII and IX as compared to group III on 30th, 45th and 60th day of experiment (Table 2 & 3).

Table 2: Comparison of T-Cell blastogenesis (Mean OD ± SE) at 15 days interval in different experimental groups in layer

Group no.	T cell blastogenesis assay on			
	15th day	30th day	45th day	60th day
I	0.125± 0.0005	0.126± 0.0006	0.130± 0.0006	0.137± 0.0006
II	0.123± 0.0002	0.124 ± 0.0005	0.126 ± 0.0004	0.136 ± 0.0005
III	0.121± 0.0007	0.122 ± 0.0005*	0.124 ± 0.0004*	0.135 ± 0.0004
IV	0.134± 0.0008	0.148 ± 0.0006*	0.156 ± 0.0006*	0.165 ±0.0003*
V	0.136± 0.0006	0.150 ± 0.0005*	0.157 ± 0.0007*	0.166 ±0.0007*
VI	0.125± 0.0008	0.130 ± 0.0003^	0.135 ± 0.0004^	0.142± 0.0004^
VII	0.127± 0.0005	0.131 ± 0.0006^	0.136 ± 0.0008^	0.143± 0.0007^
VIII	0.124± 0.0009	0.128 ± 0.0005†	0.130 ± 0.0005†	0.140 ±0.0003†
IX	0.126± 0.0006	0.129 ± 0.0008†	0.132 ± 0.0009†	0.142± 0.0006†

Superscripts are to be read column wise for mean comparison.

* P < 0.05 significant difference as compared to group I

^ P < 0.05 significant difference as compared to group II

† P < 0.05 significant difference as compared to group III

In this study, T-cell blastogenesis and B-cell blastogenesis capacity increased significantly in group IV (cow urine distillate) and group V (cow urine distillate and polyherbal extract) as compared to control group I while in groups VI and VII as compared to group II and in groups VIII and IX as compared to group III throughout the experiment. It showed that lymphocyte proliferation capacity was higher with treatment of cow urine distillate and poly herbal extract.

Table3 : Comparison of B-Cell blastogenesis (Mean OD ± SE) at 15 day interval in different experimental groups in layer

Group no.	B-Cell blastogenesis assay on			
	15th day	30th day	45th day	60th day
I	0.131± 0.0004	0.148 ± 0.0003	0.173 ± 0.0003	0.195 ± 0.0008
II	0.128 ± 0.0002	0.143 ± 0.0004*	0.170 ± 0.0005*	0.193 ± 0.0005
III	0.125 ± 0.0005	0.140 ± 0.0005*	0.168 ± 0.0004*	0.192 ± 0.0004
IV	0.135 ± 0.0004	0.160 ± 0.0006*	0.183 ± 0.0006*	0.228 ± 0.0005*
V	0.137 ± 0.0006	0.162 ± 0.0007*	0.186 ± 0.0005*	0.230 ± 0.0006*
VI	0.132 ± 0.0007	0.147 ± 0.0004^	0.177 ± 0.0005^	0.206 ± 0.0007^
VII	0.133 ± 0.0008	0.150 ± 0.0007^	0.179 ± 0.0006^	0.205 ± 0.0004^
VIII	0.130 ± 0.0003	0.144 ± 0.0004†	0.172 ± 0.0006†	0.202 ± 0.0005†
IX	0.131 ± 0.0003	0.145 ± 0.0009†	0.175 ± 0.0008†	0.203 ± 0.0007†

Superscripts are to be read column wise for mean comparison.

* P < 0.05 significant difference as compared to group I

^ P < 0.05 significant difference as compared to group II

† P < 0.05 significant difference as compared to group III

Chauhan and singh (2001) studied the immunomodulatory effect of cow urine in mice and found that cow urine enhances both T- and B-cell blastogenesis along with increased levels of IgG. Kumar et al. (2005) also reported that cow urine enhances the T- and B-cell blastogenesis by 1.81% and 2.21% respectively in broiler chicks. Similarly Garg et al. (2005) also reported the immunomodulatory effect of cow urine in layer birds by enhancing T- and B-cell blastogenesis at the rate of 28.12% and 16.6% respectively in cow urine treated group. Sonu et al. (2006) observed significant increase in both B and T cell blastogenesis in cow urine treated cells and also in combination treatments of NOEL/10^3 dose of dimethoate and cow urine. Chatterjee and Das (1997) also reported that IMMU-21 (a polyherbal formulation, which contains the extracts of different immune active plants viz. Ocimum Sanctum, Withania somnifera, Tinospora cordifolia and Emblica officinale as major constituents in their optimum concentrations) was also capable of enchancing blastogenic response of murine spleenic lymphocytes to specific antigenic challenge.

The findings showed that cow urine alone and its combination with polyherbal extract upregulates lypmphoblastogenesis in developing stages of chicks and imparted significant effect on immune status of birds.

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