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Effect of ketoprofen co-administration on immunological responses of enrofloxacin in goats

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Abstract
Antimicrobial and anti-inflammatory agents are required to be administered concurrently for a prolonged period and hence, the immunological effects on prolonged administration are required to be studied. Several studies indicate that antimicrobial and non-steroidal anti-inflammatory agents have role which affect the host immunity and thereby alter course of diseases. The present study was conducted to assess the immunological interaction of enrofloxacin and ketoprofen in goats when administered together by multiple intramuscular administration. Four groups of goats comprising of five animals in each group were selected for the study. Group I consisted of saline control, group II contained animals with antigen (Ag) control [2 ml of 7% sheep red blood cell (SRBC) Ag given on 1st day as sensitizing dose and on 10th day as challenging dose], group III consisted of animals exposed to enrofloxacin and SRBC Ag (enrofloxacin @ 5 mg/kg i.m. daily for 7 days during sensitizing and challenging period and SRBC as given in group II). Group IV consisted of Ketoprofen and Enrofloxacin when administered along with SRBC Antigen. Apart from antigen (SRBC) given in group II, enrofloxacin @ 5mg/kg and ketoprofen @ 3 mg/kg i.m. were administered daily for 7 days during sensitizing and challenge period. Blood samples were collected on 1, 7, 10, 14, 21, 28, 35 and 42 days of the experiment. The immunological parameters evaluated were haemagglutination (HA) test for humoral immune response (HIR) whereas absolute lymphocyte count (ALC) and delayed type hypersensitivity (DTH) for evaluating cell mediated immune response (CMIR). DTH was assessed by injecting 1-chloro-2, 4-dinitrobenzene (DNCB), phytohaemagglutinin-P (PHA-P) and purified protein derivative (PPD) in the skin of the neck region. The agglutinating antibody titres recorded higher titre in enrofloxacin treated group as compared to antigen treated group. Three mitogens used in the study revealed very least immunomodulatory effect when the drugs were given alone. It is concluded that these drugs when given in in multiple doses exert immunomodulatory effect on humoral immunity while only very little effect was evident on the CMIR.

Keywords: Enrofloxacin, ketoprofen, immunomodulation, goat, HIR, CMIR, PPD, PHA-P, DTH

Introduction
Antimicrobial and anti-inflammatory agents are required to be administered concurrently for a prolonged period and hence, the immunological effects on prolonged administration are required to be studied so that the assessment of therapeutic effects are to be studied thoroughly for its effective use in therapy and immunological interactions between them have been described [1]. The effect of simultaneous administration of pefloxacin and diclofenac sodium on certain natural host defence mechanisms and on specific humoral or cell medicated immune response in rabbits was studied [1]. The fluoroquinolones are popular class of antibiotics for use in a variety of infections in humans and animals. They are also known to have direct effects on the immune system [2-3]. The immunomodulatory effects of fluoroquinolones are probably due to their effects on intracellular cyclic AMP and phosphodiesterase, on transcription factors such as NF-Kappa B and activator protein 1 [3]. Enrofloxacin is metabolized into pharmacologically active metabolite, Ciprofloxacin which is also known to have modulatory effect on the immune system [2-5]. Ketoprofen possesses powerful anti-inflammatory, analgesic and antipyretic properties. When Ketoprofen is used concomitantly with primary antibacterial agent, it significantly improved recovery in gram-negative clinical mastitis in dairy cows [6]. In an another study [7], the effect of repeated ketoprofen administration during surgical castration of bulls on immune function and showed that ketoprofen has no influence on changes in acute-phase proteins and immune response. The immunological effect of ketoprofen was showed that the drug is having immune-potentiating effect on humoral immune response [8]. Looking into the above reports of immunological properties of enrofloxacin and ketoprofen studied by various workers, the present study was undertaken with the objective to assess the

immunological interactions of both these drugs when administered together.

Materials and Methods

Experimental animals: In the present study, 20 clinically healthy Black Bengal female goats (*Capra hircus*) between 20 to 24 months of age weighing 18-22 kg body weight were used.

Experimental design: For conducting immunological study, clinically healthy goats were divided into four groups consisting of five animals in each group. Details of treatment given to different groups are summarized as given below.

Group I: Saline Control Group.

Group II: Antigen Control Group. 2 ml of 7% sheep red blood cell (SRBC) suspension intravenously (i.v.) given in each goat on the first day of experiment (sensitizing dose) and on the 10th day of experiment (challenging dose).

Group III: Enrofloxacin + Antigen Group. Apart from antigen (SRBC) given as in Group II, enrofloxacin was administered @ 5mg/kg. i.m. daily for 7 days during sensitizing and challenge period.

Group IV: Ketoprofen + Enrofloxacin + Antigen Group. Apart from antigen (SRBC) given in group II, enrofloxacin @ 5mg/kg and ketoprofen @ 3mg/kg i.m. were administered daily for 7 days during sensitizing and challenge period.

Administration of Drugs: Enrocin®, an injectable commercial preparation containing Enrofloxacin (10%) in concentration of 100 mg. ml^{-1} marketed by Ranbaxy Laboratories Limited, India was used in the present experiment. Ketoprofen, an injectable commercial preparation marketed under trade name of Neoprofen® by Ranbaxy Laboratories Ltd. India was used. Each ml of Neoprofen contains 100 mg of ketoprofen. Enrofloxacin (5mg/kg) and Ketoprofen (3 mg/kg) was administered i.m. for 7 days in healthy goats during period of pre and post challenge.

Collection of biological fluids and their timings: Blood samples were collected on day 1, 7, 10, 14, 21, 28, 35 and 42 days of the experiment without anticoagulant for serum separation. For Haemagglutination test (HA) and absolute lymphocyte count also, blood samples were collected on same days as mentioned above with anticoagulant.

Analysis of Immunological parameters

Preparation of buffers: Phosphate buffer saline (PBS) was prepared using NaCl, KCl, $Na_2HPO_4.2H_20$, KH_2P04 and distilled water, pH 7.2 to 7.4 as per a prescribed method [9]. PBS was used for reconstitution and preparation of sheep red blood cells (SRBC). Alsever's solution was prepared using dextrose, sodium citrate, sodium chloride, citric acid and distilled water. Equal volume of Alsever's solution and anticoagulant was used for collection of sheep blood.

Mitogen used for cutaneous basophilic hypersensitivity reactions (delayed type hypersensitivity): 1% DNCB (1-Chloro-2, 4-dinitrobenzene) solution was prepared in acetone (10 mg/ml). PHA-P (Phytohaemagglutinin-P) was used as mitogen for cutaneous basophilic hypersensitivity reaction. A concentration of 1 mg/ml of PHA-P in PBS solution was prepared. PPD (Tuberculin) @ 10 IU/0.1 ml was used.

Assessment of immune response after administration of different drugs: Humoral immune response (HIR) was assessed by haemagglutination (HA) test. HA test was performed in the sera of test and control goats. The anti-SRBC antibody titres were measured using micro-titration technique [10]. The HA pattern was read with the aid of reading mirror and result of HA titre was recorded reciprocal of the highest dilution showing 100% HA and expressed a $\log_2$ HA titre/0.5 ml of goat's serum.

Cell-medicated immune response (CMIR) was assessed by Delayed type hypersensitivity (DTH) reactions which included DNCB, PHA-P and PPD Skin sensitivity tests. DNCB test was done as per a previously described [11]. 0.25 ml of DNCB (10mg/ml) in acetone vehicle was applied on right side. On left side, 0.25 ml of acetone was applied which served as control. DNCB was applied on 5th day and challenged on 15th day of experiment by applying 0.25 ml of DNCB in acetone on right side and 0.25 ml acetone on the left side at the same site of first application. The skin thickness was measured with the help of slide caliper at 4, 8, 12, 24, 48, 72 and 96 h during pre- and post-challenge. The CMI response was calculated by subtracting the thickness of right side from left side.

PHA-P skin sensitivity test was done as per a previously described method [12]. 0.1ml of PHA-P (1mg/ml) in 0.1ml of PBS was injected intradermally on the right side of neck. The left side received 0.1ml of sterile PBS and served as control. The PHA-P stimulation index was calculated as the difference in swelling on PHA-P injected and PBS injected site with the help of slide caliper on 4, 8, 12, 24, 48, 72 and 96 h. Purified protein derivative (PPD) Skin Sensitivity test was done as per a previous method [13]. The results were expressed as the difference of swelling on PPD injected site and PBS injected site at 4, 8, 12, 24, 48, 72 and 96 h post injection during pre- and post-challenge periods.

Cell-medicated immune response was also assessed by absolute lymphocyte count as the method described in Schalm's veterinary Haematology: -

Absolute lymphocyte count = (Total no. of lymphocyte ÷ 100) × Total no. of leukocyte.

Statistical analysis: The effects of drug on immune response at different time intervals and on various days of post treatment in groups was done by random design (CRD).

Results and Discussion

Humoral Immune Response (HIR)

Effects of different drugs such as enrofloxacin alone and enrofloxacin given together with ketoprofen on humoral immune response in goats were recorded using sheep red blood cell (SRBC) as an indicator of humoral immunity. Table 1 showed the humoral immune response of enrofloxacin and ketoprofen against – SRBC antigen (CRD mean±S.E.) to HA antibody titre (log 2 Value) in goats.

Table 1: Humoral immune response (HIR) of drug against SRBC antigen to antibody titre (Log 2value) in goats

Group	CRD mean±SE
1. Saline Control	0.30103^{A}±0
2. Antigen Control	0.88050^{AB}±0.19901
3. Enrofloxacin + Antigen	1.65565^{B}±0.34735
4. Ketoprofen + Enrofloxacin + Antigen	1.85885^{B}±0.37709

Means with different superscript within column differ significantly (p<0.05)

The study revealed that there is no immunomodulatory effect on humoral immunity by enrofloxacin when given alone but in case of combined administration of enrofloxacin with ketoprofen showed immunomodulatory action on humoral immunity. The HA antibody titre were recorded in enrofloxacin and ketoprofen together treated group (1.85885±0.37735) produced significantly higher titer followed by non-significantly higher titer by enrofloxacin (1.65565±0.34735). Therefore, it is suggested that only significant immunomodulatory effect has occurred on humoral immune response by simultaneous administration of enrofloxacin and ketoprofen. The above findings are not in agreement with the results of a previous study [1] who observed that the simultaneous administration of pefloxacin and diclofenac produced a marginal suppression of humoral immune response in rabbits. Pefloxacin and ciprofloxacin were reported to alter the humoral immune response of mice against SRBC [14]. Aspirin and acetaminophen suppressed the serum neutralizing antibodies in human beings [15].

Cell-medicated immune response (CMIR)

Effects of different drugs such as enrofloxacin and ketoprofen given separately and in combination on CMIR in goats after multiple i.m. administration were observed by using three different mitogen (DNCB, PHA-P and PPD / Tuberculin) and absolute lymphocyte count as indicator of cell-medicated immunity.

The result of absolute lymphocyte count (per cubic millimetre) revealed that simultaneous administration of enrofloxacin and ketoprofen caused an apparent decrease of lymphocyte count (5220±152.720), which was not statistically significant with antigen control (5400±150.80) but alone administration of enrofloxacin (4457±36.31) caused significant decrease of lymphocyte count with antigen control (5400±150.80).

Table 2: Cell-medicated immune response (CMIR) of drugs to absolute lymphocyte count (per cubic millimetre).

Group	CRD mean±S.E (Per cubic millimetre)
1. Saline Control	4516^{a}±15.890
2. Antigen Control	5400^{b}±150.800
3. Enrofloxacin + Antigen	4457^{a}±36.310
4. Ketoprofen + Enrofloxacin + Antigen	5220^{b}±152.720

Means with different superscript within column differ significantly (p<0.05)

Table 3 represents the results of CMIR with regard to DNCB, PHA-P and PPD tuberculin mitogens in pre-challenge and post challenge. With respect to DNCB mitogen, the results indicate that there is non-significant decrease in skin thickness in enrofloxacin treated group (0.7631±0.0529 mm) and non-significant increase in skin thickness in enrofloxacin with ketoprofen treated group (1.1697±0.1682 mm) as compared to both the control groups in the pre-challenging period. There is significant decrease in skin thickness in enrofloxacin treated group (0.8008±0.0515 mm) and non-significant increase in skin thickness in enrofloxacin with ketoprofen treated group (1.2148±0.1618 mm) as compared to both the control groups in post-challenging period of experiment.

With regard to PHA-P mitogen, the study showed no immunomodulatory action on cell-medicated immunity by these drugs. Non-significant observations were found by these drugs in comparison to both negative and positive controls.

Table 3: Cell medicated immune response (CMIR) of drugs to DNCB, PHA-P and PPD/tuberculin mitogens in goats.

Pre-challenge (5th day)

Group	CRD mean±S. E		
	DNCB	PHA-P	PPD/tuberculin
1. Saline Control	1.0440^{a}±0.0733	0.7988^{a}±0.0724	0.5734^{a}±0.0331
2. Antigen Control	1.0442^{a}±0.0945	1.0222^{a}±0.0724	0.6947^{b}±0.0876
3. Enrofloxacin + Antigen	0.7631^{a}±0.0529	0.8768^{a}±0.0628	0.6100^{a}±0.0686
4. Ketoprofen + Enrofloxacin + Antigen	1.1697^{b}±0.1682	1.1068^{a}±0.2036	0.9422^{c}±0.0648

Means with different superscript within column differ significantly (p<0.05)

Post-challenge (15th day)

Group	CRD mean±S. E		
	DNCB	PHA-P	PPD/tuberculin
1. Saline Control	1.2308^{a}±0.0995	1.0485^{a}±0.0798	0.6628^{a}±0.0517
2. Antigen Control	1.2537^{a}±0.1140	1.774^{a}±0.1044	0.9337^{bc}±0.0641
3. Enrofloxacin + Antigen	0.8008^{b}±0.0515	0.9505^{a}±0.0699	0.7577^{ab}±0.0834
4. Ketoprofen + Enrofloxacin + Antigen	1.2148^{a}±0.1618	1.1314^{a}±0.2025	0.9922^{c}±0.0647

Means with different superscript within column differ significantly (p<0.05)

For PPD tuberculin mitogen, the results indicate increase in cutaneous basophilic hypersensitivity (CBH) response was observed in enrofloxacin + ketoprofen treated group (0.9422±o.0648 mm) produced significantly higher that showed immunomodulatory action on cell-medicated immunity in pre-challenged period of experiment.

The DNCB and PHA-P skin sensitivity tests do not suggest significant immunomodulatory effect on cell medicated immune response by the drugs used in the present study. In contrast, PPD skin sensitivity test showed significant immunomodulatory effect on CMIR in pre- challenge period of experiment in enrofloxacin + ketoprofen treated group only. Similar results were reported in a previous study [16] who observed that pefloxacin, ciprofloxacin and ofloxacin

significantly inhibited mononuclear leukocyte proliferation in response to mitogen phytohemagglutinin. But, an another study [17] reported that ciprofloxacin neither diminished nor enhanced mononuclear cell proliferation. In an another study [1], it was found that simultaneous administration of pefloxacin and diclofenac did not affect the natural host defence mechanism or immune response to the known antigen significantly as well as enrofloxacin and ketoprofen also did not affect the natural non-specific host defence mechanism, which are essential for warding off infections. Simultaneous administration of enrofloxacin caused an apparent decrease of absolute lymphocyte count, whereas significant decrease was observed in absolute lymphocyte count in enrofloxacin and ketoprofen treated groups and their respective positive control. Pefloxacin, ciprofloxacin and ofloxacin have been reported to inhibit mononuclear leukocyte proliferation in response to mitogen Phytohemagglutinin [16].

Based on the immunological interactions of enrofloxacin and ketoprofen in the present investigation, it is concluded that enrofloxacin can be safely and effectively used in combination with ketoprofen for treating mild to serve systemic infections, which is generally associated with pain and other inflammatory conditions.

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