ESTIMATION OF C-REACTIVE PROTEIN AND TOTAL SERUM IMMUNOGLOBULIN CONCENTRATIONS AFTER UTERINE IMMUNOMODULATION IN REPEAT BREEDING COWS

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Received: August 4, 2015; Accepted: September 2, 2015

Abstract: Twenty one repeat breeding cows were screened for subclinical endometritis and they were assigned equally to one control group and two treatment groups. Cows in the control group were infused with 50 ml of normal saline intra-uterine. Out of the two treatment groups, cows in group I were treated with 20 ml of colostrum as intra uterine medication. Cows in treatment group II were infused with 10 ml of non pathogenic E.coli (pure attenuated culture obtained from Himedia Product code – ATCC 25922). C-reactive protein concentrations and total immunoglobulin concentrations were calculated on day 0, day 7th, day 14th and day 21st of estrus cycle from the blood sample. The estimation of C-reactive protein in all the experimental groups revealed a range between 1.2 – 4.8 mg/dl with the negative values obtained for 21 day of estimation indicating improvement of uterine health. Comparison of immunoglobulin values for different days in all the treatment protocol revealed a significant (p<0.05) difference within various days of sampling.

Key words: C-reactive protein, Serum immunoglobulin, Cow

INTRODUCTION

Repeat breeding problem is a major concern which affects the farming community by causing conception failure in cows even after repeated inseminations. The repeat breeder animal is defined as sub fertile animal which failed to conceive after three or more services in absence of any obvious pathological disorders of the genital tract and cyclicity. The predisposing causes may be due to genetic, hormonal imbalance, bad management, infectious and noninfectious factors etc [1]. Traditionally various hormones has been advocated to contain repeat breeders provided nutrition and management are adequate [2]. In Indian scenario subclinical endometritis might be a major etiology of repeat breeding and routine use of antibiotics are usually recommended either parentrally or as intra-uterine medications. Invariably, indiscriminate use of antibiotics might develop insensitivity against certain microbes leading to treatment failure. The need for an alternative form of treatment is imperative to ameliorate repeat breeding instead of conventional antibiotic treatment. Animals undergoing external or internal challenge to their state of health mount a vigorous response including activation of both the innate and acquired immune systems. The innate immune system which covers those aspects of the host defense mechanisms not dependent on specific response, such as production of antibody, not only stimulates leukocyte activity but also effects many aspects of the host’s metabolic processes [3]. The varied reactions of the host to infection, inflammation, or trauma are collectively known as
the acute-phase response (APR) and encompass a wide range of pathophysiological responses such as pyrexia, leukocytosis, hormone alterations, and muscle protein depletion combining to minimize tissue damage while enhancing the repair process [4].

C-reactive protein (CRP) is used mainly as a marker of inflammation and infection. Measuring and charting CRP values can prove useful in determining disease progress or the effectiveness of treatments. Viral infections tend to give a lower CRP level than bacterial infection. Infusion of E. coli LPS as uterine immunomodulator not only increases the influx of neutrophils into the uterine lumen but also eradicates uterine infection that may be associated with mild to severe form of endometritis which ultimately results in cyclic non-breeding [5]. The present treatment protocol does not involve use of antibiotics for combating the microbial infection of uterus, which in due course, can lead to development of bacterial resistance, minimizing the cost of treatment and diminishing uterine defense mechanism. Hence, an alternative therapy such as intra-uterine defense stimulators like of colostrum and non-pathogenic E. coli, has been tried in confirmed cases of repeat breeding associated with bacterial endometritis [6]. Therefore, the present investigation was designed to study the effect of administration of colostrum and E. coli attenuated culture, in repeat breeding cases and comparing it with effect of administering normal saline solution in obstinate repeat breeding cases, so as to evolve a protocol, which will be beneficial in field conditions to combat the menace of repeat breeding.

MATERIALS AND METHODS

The screening of repeat breeding cows was based on the following criteria laid down by Laing et al. [7] and whiteside test [8]. A total number of twenty one repeat breeding cows selected after meticulous screening were allotted randomly into three equal groups (n=7). The animals in control group received 50 ml of normal saline as intra-uterine medication. Cows in group -1 and group -2 lavaged with 20 ml of fresh bovine colostrums and 10 ml of non-pathogenic E. coli in sterile saline solution. Blood samples were collected from all the animals on day 0, 7, 14 and 21 of estruses cycle. Ten ml of blood was collected aseptically from jugular vein of animals and was used to harvest serum. The serum thus collected was stored in sterilised vial at -20°C for estimation of acute phase protein (C-reactive protein). This was estimated by performing slide test for C–reactive protein using RHELAX-CRP kit by both qualitative and semi-quantitative methods. The qualitative method based upon the simple agglutination where one drop of test specimen was mixed with one drop of RHELAX-CRP latex reagent. The mixture was uniformly mixed and if agglutination occurs within two minutes the sample was taken as positive.

In the semi quantitative method those samples which were found positive by qualitative methods serial dilutions were prepared by using isotonic normal saline of test specimen, found positive in the qualitative method as 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and so on. Each dilution of test specimen was pipette onto separate reaction circles present in the strip provided by the kit. One drop of RHELAX-CRP latex was added to the drop of test specimen on the slide. Using a mixing stick the test specimen and latex reagent was mixed uniformly over the entire circle. Samples observed for agglutination within two minutes to be taken as positive (Figs. 1,2).

Agglutination in the highest serum dilution corresponds to the approximate amount of CRP in mg/dl present in the test specimen. Concentration of CRP can be calculated as follows. CRP (mg/dl) = SxD, where S = sensitivity of the reagent i.e. 0.6mg/dl, D = highest dilution of serum showing agglutination

The total immunoglobulin concentration of blood serum collected from all the experimental animals were processed for immunoglobulin estimation as per the method described by Chauhan [9].

In a test tube 5.7 ml of ammonium sulphate - sodium chloride mixture was taken and to 0.3 ml of clear serum sample was over layered. The contents were mixed gently and placed on ice bath for 15 minutes. Then the test tube was centrifuged at 3000 rpm for 10 minutes at 4°C and the supernatant was discarded. This process was repeated twice and finally the contents were dissolved in 2ml normal saline solution.

To the dissolved contents, 5 ml of Biuret reagent was added and kept for 10 minutes at room temperature. A blank was prepared with 2 ml of normal saline and 5 ml of reagent in a test tube and
Fig 1: The diagnostic plate provided by TULIP Diagnostic Pvt Ltd for the qualitative and semi quantitative estimation of CRP concentration.

Agglutination absent indicating test sample is negative.

Fig 2: Indicating test positive and negative sample.

Agglutination present indicating test sample is positive.
marked as B (Blank). The standard was prepared by placing 2 ml of 0.15 % bovine serum albumin and 5 ml of biuret reagent in a test tube and marked as S (standard). The absorbance of transmittance of test and standard was read against blank set at ‘zero’ at 555 nm wave length in spectrophotometer. The total serum immunoglobulin was calculated by following equation.

\[
\text{Total serum immunoglobulin (gm/dl)} = \frac{\text{OD of test}}{\text{OD of standard}} \times \text{concentration of standard}
\]

**RESULTS**

In normal saline infused group, six showed agglutination reaction out of seven samples and the values ranged between 1.2 – 4.8 mg/dl. The 7th day analysis showed its presence in four samples and registered a range between 1.2 – 2.4 mg/dl. The 14th and 21st day estimation did not show any quantifiable reaction except in one (1.2 mg/dl) on 14th day. In colostrum treated cows, the pre-treatment (day 0) estimation showed six positive cases out of seven samples which averaged between 1.2 – 4.8 mg/dl. The frequency of CRP values further depleted on day 7th (1.2 – 2.4 mg/dl) and 14th day (1.2 mg/dl) sampling. However, none of the serum samples were positive for CRP on 21st day of collection. In the nonpathogenic *E. coli* infused cows, the CRP value ranged between 1.2 – 4.8, 2.4 and 1.2 mg/dl for day 0, 7, 14 respectively. However, the 21st day sampling is negative for CRP for all the cows. Only the normal saline and colostrum infused group showed significant difference (p<0.05) within different days of sampling. Non-pathogenic *E. coli* treated group didn’t reveal any significant difference within the groups (Table 1; Fig. 3).

The serum Ig concentration value (gm/dl) for day 0, 7, 14 and 21 of sampling in normal saline treated cows are given in Table 2. Comparison of Ig values for different days revealed a highly significant (p<0.05) difference within group. Test of significance between 0 day against 7th and 14th day showed highly significant (p<0.01) difference with respect to Ig value. However, the 7th day value was significantly higher (p<0.05) compared to 21st day. On the contrary, other interactions of Ig values were not significant. The colostrum treated group registered a value of 2.03±0.22, 4.05±0.33, 3.20±0.21 and 2.30±0.21 (gm/dl) concurrently on the same days of sampling. Analysis of variance registered a highly significant (p<0.01) difference within values on different days of sampling. Comparison of sampling value of Ig showed highly significant (p<0.01) difference against 0 Vs 7, 0 Vs 14 and 7 Vs 21 days. Day 14 value was significantly higher (p<0.05) than the 21st day. The interaction of remaining Ig values between days did not reveal any significant difference. In the nonpathogenic *E. coli* infused group, the estimated Ig (gm/dl) values were mentioned in Table 2. Analysis of variance of values

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>TEST PROTOCOLS</th>
<th>NORMAL SALINE</th>
<th>COLOSTRUM</th>
<th>E.COLI</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAYS</td>
<td>0</td>
<td>7</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>2.23 ± 0.71a</td>
<td>1.37 ± 0.66b</td>
<td>0.17 ± 0.17a</td>
<td>2.23 ± 0.61a</td>
</tr>
<tr>
<td>F-VALUE</td>
<td>3.27a</td>
<td>3.88*</td>
<td>1.19</td>
<td>3.27a</td>
</tr>
</tbody>
</table>

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</thead>
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<tr>
<td>DAYS</td>
<td>0</td>
<td>7</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>Total Ig (g/dl)</td>
<td>2.22 ± 0.24b</td>
<td>3.74 ± 0.23b</td>
<td>3.05 ± 0.23b</td>
<td>2.45 ± 0.20b</td>
</tr>
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on different days of sampling recorded a highly significant (p<0.01) difference within various days of collection. Test of significance between values at different days revealed that the 7th and 14th day sampling had significantly (p<0.01) higher Ig concentration than day 0. Similarly 21st day value was significantly lower (p<0.01) than 7th day value with respect to Ig concentration. Other comparisons between different days of sampling did not record any significant difference (Fig. 4).

**DISCUSSION**

In all the groups it is evident that the CRP values were negative on 21st day of sampling. Further the frequency also showed concurrent decrease with the progression of days of sampling, indicating clinical recovery of the cows. C-reactive protein is one of the most important protein component synthesized by liver which rises during acute inflammatory process such as infection. It is a useful marker of inflammation and infection. It also plays an important role to evoke innate immunity and helpful in getting rid of infection. Its value is more useful in bacterial infections and can be used as useful marker for diagnosis and recovery [10]. The CRP value in the present study is in partial agreement with the study of Morimatsu [10], who reported a concentration of 39.8±47.5 µg/ml in normal cows. In acute inflammation, the CRP values rise dramatically within 48 hrs. and may go 4 to 5 fold than the normal value. In the present context the selected repeat breeding cows might have low degree of infection which could not have induced potent inflammatory reactions to reflect significant CRP rise in the blood. The quantification of CRP might provide diagnostic and prognostic information for monitoring herd health [11] but its usefulness is limited except in acute inflammatory conditions. The discrepancy of CRP value than the reports by other workers might be due to difference in methodology employed.

Normal saline as an intra-uterine infusion have been experimented on earlier occasions in cows and mares.
The significant rise in total Ig might be nonspecific but it could be presumed that the infective agent might have diluted and accompanied by increase in blood supply. This physical process of dilution might have stimulatory effect on circulatory Ig level. The significant increase in 7th and 14th day of Ig value in colostrum treated cows might be due to immuno-stimulatory reaction caused by colostrum application. Autologous serum [12] has been a method of intra uterine treatment in cases of repeat breeding and endometric cows. Colostrum being a body fluid is similar to serum with respect to Ig concentration. Colostrum is very rich in IgG, IgA and IgM of which IgG (80mg/ml) is predominant followed by IgA. The increase in Ig concentration in initial phase of infusion might be due to their inhibitory property against infective agents. The increase in immunoglobulin following colostrum treatment in blood might be due to antigenic response of antibody present in the colostrum which stimulated the production of plasma cells from lymphocytes. Further, it can be suggested that bacterial elimination by the action of passive immunity and antigenic stimulation might be a possible cause of elevation of Ig in the serum.

In nonpathogenic E. coli treated group the elevation of Ig is due to presence of lipopolysaccharides on its cell membrane. Lipopolysaccharides have got potent antigenic property and its effect on proliferation of PMN cells in the uterine lumen with production of more antibodies with stimulation of both humoral and cellular immunity, have been established [13]. Use of oyster shell glycogen and E. coli LPS could stimulate nonspecific and specific immunity in uterine tube. Nanda and Thaperwal [14] reported a systemic immuno-modulation by intra uterine application of E. coli and P. granulosum. They postulated that local immuno-modulation leading to rise in Ig concentration were responsible for phagocytosis and elimination of bacterial infection.

The total Ig of the body relates to the subtotal specific and nonspecific immunity status of the body. Antibodies are member of a family of proteins called immunoglobulins. They are complex glycoproteins. The mother of all immune cells are a segment of total lymphocytes dedicated to immune function by way of imparting both cellular and humoral immunity [15]. The therapeutic efficacy of different intrauterine therapies, like normal saline, colostrum and nonpathogenic E. coli medication have got mild systemic immunomodulatory effect. The use of the diagnostic kit for C-reactive protein may act as a helping hand for the farmers at the door step which enable them for monitoring the reproductive status of the dairy herdmode of the treatment with non-conventional drugs, might act as an alternative choice in treating repeat breeders against routine use of antibiotics, which has got many limitations.

REFERENCES