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Research Paper

COLLAGENASE VALIDATION ASSAY FOR THE ASSESSMENT OF EFFICACY AND DOSE ASSESSMENT OF BACTERIALCOLLAGENASE FOR THE TREATMENT OF RFM IN COWS

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The collagenase administration through umbilical artery is the effective treatment for Retained Fetal Membrane (RFM) in dairy cows. RFM was treated with collagenase enzyme through jugular vein as it is easy route than previous study of experimenting on umbilical arteries which is very difficult in a delayed case in field conditions in bovines. The study was conducted in placental tissues of bovines that are presented within 12 to 24 hours after parturition to Obstetrics Unit of Madras Veterinary College, Chennai to determine the efficacy of enzyme and dose effectiveness prior to actual treatment with field cases for therapeutic purposes. The experimental animals were divided in to four different treatment groups (Gp I:n=7; Gp II:n=15; Gp III:n=15 and Gp IV:n=15) and placental tissue samples were collected accordingly from all groups.The dose of 1800 CDU collagenase had significantly higher (P<0.01) concentrations of placental tissue hydroxyproline (35.00 ± 0.63 to 36.31 ± 0.31 µg/ml) and placental tissue total proteinrelease (1.68 ± 0.02 to 1.73 ± 0.02 g/dl), than the remaining groups. The data were collected, compared and analyzed; further, the findings showed that 1800 CDU dose was selected for the RFM treatment

Keywords: Placental tissues collagenase validation assay, Hydroxyproline, Total protein, Serotonin values, cows.

INTRODUCTION

Retained Foetal Membrane (RFM) is one of the most important post parturient disease (Stephen, 2008), leading to reproductive problems and economic losses in dairy industry (Pathak *et al.,* 1991). The incidence of RFM ranges from 3 to 15% following normal parturition in dairy cows (Sheetal *et al*., 2015). A variety of methods have been used in the treatment of RFM, which includes manual removal and/or administration of oxytocin, $PGF_{2\alpha,}$ antibiotics, immune modulators etc. (Amin *et al.,* 2013), although the efficacy of these treatments are questionable (Eiler, 1997). Hence, bacterial collagenase from

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Clostridium histolyticum was used for the treatment of RFM as it could degrade several types of collagen (Azawi, 2013).

Based on these and to ascertain the efficacy of collagenase as this is pre requisite before commencing treatment of RFM with collagenase protocol, this *in-vitro* study was formulated to determine the effect of collagenase on placental tissues.

MATERIALS AND METHODS

Placental tissue samples from fifty two healthy and parous cows less than 10 years of age, presented to the Large Animal Obstetrics Unit, Teaching Veterinary Clinical Complex, Madras Veterinary College,and Chennai-7 with RFM were utilized for the study.Seven healthy cows with normal calving and shedding of placenta were served as group I (control). Thirty cows with unassisted calving followed by retained foetal membranes between 12 and 24 h interval were selected and randomly allotted into groups II and III of fifteen each. Fifteen cows with difficulty in parturition followed by RFM were considered as group IV.

Materials Collected and Prepared

Preparation of Collagenase Solution for Treatment

A single dose of 2,00,000 unitsof collagenase stock powder was prepared with 166 mg of collagenase powder (Sigma Aldrich, USA), 40 mg of calcium chloride and 40 mg of sodium bicarbonate kept in 15 ml of sterile screw capped vials and stored at -20 pC. Before 2 to 3 hof treatment, the collagenase stock powder was dissolved in 10 ml of normal saline and reconstituted with 1 litre of normal saline (Plate) and kept ready for administration (Fecteau *et al.,* 1998).

In vitro Collagenase Validation Assay

Collection of Placental Tissue Sample

Placental tissue samples were collected on day 0 (within12 to 24 h after parturition) in all the groups and stored at -20 pC until the estimation of hydroxyproline and total protein to assess *in-vitro* effect of different doses of collagenase and serotonin concentrations in the placental tissues (Kankofer *et al.,* 1998; and Dehghan *et al.,* 2007).

Preparation of Collagenase Stock Solution for *in vitro* Collagenase Validation Assay

Collagenase stock solution for *in-vitro*collagenase validation assay was prepared with 166 mg of collagenase powder (2,00,000 CDU) dissolved in 20 ml of normal saline (Plate).

Preparation of Placental Tissue Samples for Collagenase Validation Assay

Three grams of placenta in each group was taken in four individual flasks. Flask one was added with 15 ml normal saline which acted as control, remaining three flasks were added with 60 µl (0.66 mg or 800 CDU), 120 µl (1 mg or 1200 CDU) and 180 µl (1.5 mg or 1800 CDU) of collagenase stock solutions, respectively. All the flasks were incubated at 39 pC for 4 h and fluids were filtered with 10 cm x 10 cm three layered gauze sponges filled in sterile plastic vialsand stored at -20 pC until estimation of placental tissue hydroxyproline and total protein concentration (Fecteau *et al.,* 1998).

Data on release of hydroxyproline and total protein in response to the treatment with collagenase *in-vitro* were collected, compared and analyzedas per the standard procedure outlined by Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

Collagenase Validation Assay

Hydroxyproline

The hydroxyproline concentration significantly (P<0.01) increased with increased doses of collagenase in all the groups (Table). These results revealed that the higher doses of collagenase as 1800 CDU was responsible for the release of significantly (P<0.01) higher concentration of placental tissue hydroxyproline (35.00 ± 0.63 to 36.31 ± 0.31 μg/ml) in all the groups. These findings were in agreement with the observations of Fecteau *et al*. (1998) that the maximal hydroxyproline release was achieved with 1800 CDU collagenase dose (24 μg/ml) than the control group (0.04 μg/ml) cows.

Similar dose dependent trends of increased release of placental tissue hydroxyproline concentration was observed by Eiler (1992) who reported that the concentration of hydroxyproline release was 1.7 ± 0.2, 4.5 ± 0.5, 5.5 ± 0.3, 6.5 ± 0.8 and 12.0 ± 3.4 mg/ml with 10.0, 15.0, 30.0, 60.0 and 120.0 CDU dose of collagenase, respectively with 3 gm of bovine placental tissue incubated for 6 h. Further, variations of hydroxyproline concentration or collagenase like enzyme activity recorded in the incubated media depends on the method or procedures utilized to analyze the placental tissue samples; which agreed with the observations of Dehghan *et al.* (2007) that the placental tissue samples of non RFM and RFM cows had the concentration of collagenase like enzyme specific activity as 2.46 ± 0.47 and 3.60 ± 0.84 u/mg of protein, respectively and collagenase activity as 1.74 ± 0.23 and 1.47 ± 0.38 μg/ml, respectively.

The result obtained in the present study was in contrast to the observation of Haffner *et al.* (1998) that the hydroxyproline concentration was 671 ± 71.3 μg/ml in equine placenta with 1200 u/ml of collagenase incubated for 4 h. These might be the variations in the species, where the collagenase was 3.7 times more effective in digesting the equine placenta (Haffner *et al.,* 1998). Further, it has been reported that the maximum rates in degradation of type I and II collagens showed marked species variations in human, bovine and rodents (Harris and Vater, 1982; and Brodsky and Eikenberry, 1982). The concentration of collagen types in the placenta and collagen biochemical variations between species might account for the elevated concentration of placental tissue hydroxyproline (Fecteau *et al.,* 1998) in the present study.

Total Protein

The placental tissue total protein concentration

Table: Mean (± SE) in vitro Placental Tissue Concentration of Hydroxyproline and Total Protein During Collagenase Validation Assay

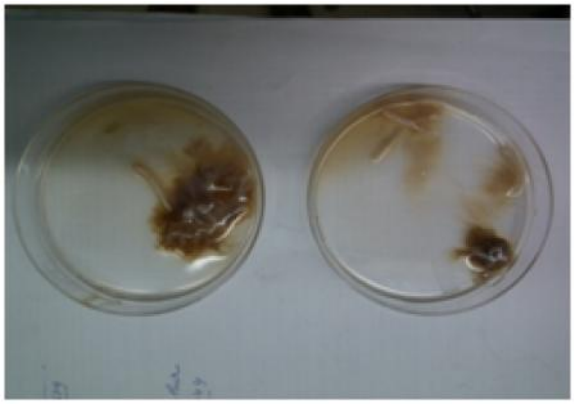
Groups/ Collagenase (CDU)	Hydroxyproline (μg/ml)				Total Protein (g/dl)			
	Control	800	1200	1800	Control	800	1200	1800
I	7.26±0.01^{aA}	17.31±0.70^{aB}	27.07±0.51^{aC}	35.00±0.63^{aD}	1.13±0.01^{aA}	1.16±0.01^{aB}	1.35±0.01^{aC}	1.73±0.02^{aD}
II	7.23±0.01^{aA}	17.87±0.17^{aB}	27.35±0.33a^{C}	36.31 ±0.31^{aD}	1.16±0.10^{aA}	1.21±0.05^{aB}	1.32±0.01^{aB}	1.72±0.02^{aC}
III	7.33±0.02a^{A}	18.28±0.16^{aB}	25.92±0.32^{aC}	35.51±0.61^{aD}	1.17±0.01^{aA}	1.19±0.01^{aA}	1.32±0.18^{aB}	1.68±0.02^{aC}
IV	7.30±0.17^{aA}	18.10±0.16^{aB}	27.02±0.50^{aC}	35.62±0.62^{aD}	1.19±0.01^{aA}	1.18±0.03^{aA}	1.36±0.03^{aB}	1.72±0.02^{aC}

Note: Means bearing different superscripts (A-B) in each row differ significantly (P<0.01); Means bearing different superscripts (a-b) in each column differ significantly (P<0.01).

significantly (P<0.01) increased with increasing doses of collagenase in all the groups as that of placental tissue hydroxyproline concentration in the present study (Table). These results, revealed that the higher doses of collagenase at 1800 CDU was responsible for the release of significantly (P<0.01) higher concentration of placental tissue protein (1.68 ± 0.22 to 1.73 and 0.02 g/dl) in all the groups. These findings were in agreement with the observations of Fecteau *et al.* (1998) that *in-vitro* collagenase validation assay concentration of placental tissue protein in human,equineandbovineplacenta were 1.01 ± 0.14, 0.83 ± 0.04 and 1.14 ± 0.19 g/dl with saline 1.16 ± 0.18, 1.65 ± 0.20 and 1.49 ± 0.14 g/dl, respectively with a dose of 1800 CDU collagenase.

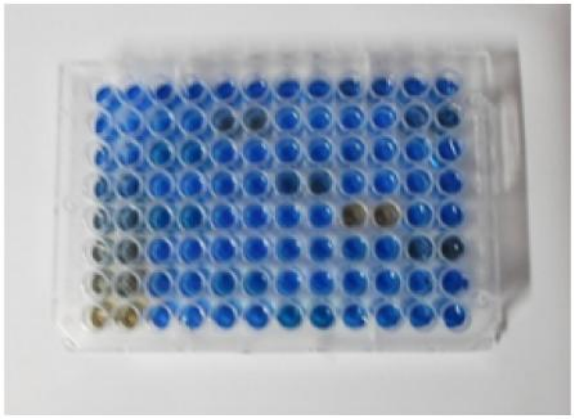
The present findings were in contrast to the observations of Sharpe *et al.* (1989) that the concentration of placental tissue total protein were 400.4 ± 17.1 and 578. ± 18.1 mg/gm on day 2 postpartum in normal calving followed by non RFM and RFM cows, respectively. Similar observations were made by Sharpe *et al.* (1990) that the concentration of placental tissue total protein at 2 and 12 h of postpartum in RFM cows were 578.1 ± 18.5 and 526.0 ± 18.5 mg/gm dry weight,

Plate: Collagenase Validation Assay

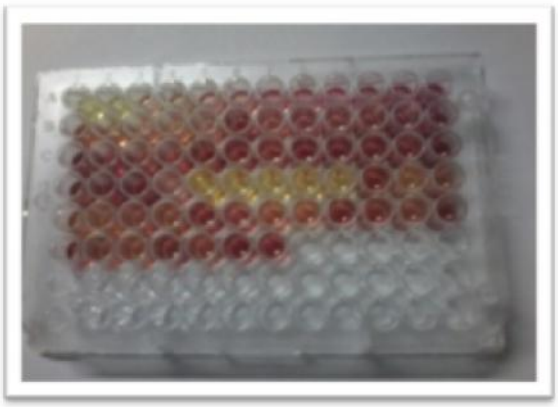


(a) Placental tissue with normal saline (control)

(b) Placentaltissue with collagenase



(c) Placental tissue total protein estimation by Bradford method



(d) Placental tissue hydroxyproline estimation with ELISA kit

respectively and immediately after placental expulsion in non RFM cows was 437.5 ± 36.2 mg/gm dry weight; Dehghan *et al.* (2007) recorded that the concentration of placental tissue total protein at 12 h after parturition in non RFM and RFM cows were 0.079 ± 0.15 and 0.41 ± 0.08 mg/ml, respectively in cows.

The variations of placental total protein concentration in the present study suggests that the proteolytic mechanisms in the cotyledon of the cows with RFM has less activity than the cows without RFM (Sharpe *et al.,* 1990), which was related to infiltrations of inflammatory cells (Neutrophils and Macrophages) in the retained placenta (Al-Sadi *et al.,* 1994). Further, these variations might be due to the placental tissues collected on the day or time of postpartum, method applied in the estimation of total protein and the involvement of specific foetal collagen proteolytic activity as essential to release the foetal membrane after parturition (Gross *et al.,* 1985).

Based on these findings, 1800 CDU/ml collagenase as equivalent of 2,00,000 units/lit has been fixed as a dose for the treatment of RFM (group III and IV) in the present study. The dose of 1800 CDU collagenase had significantly higher ($P<0.01$) concentrations of placental tissue hydroxyproline and placental tissue total protein release. Group I had significantly lower ($P<0.01$) placental tissue serotonin concentration and group IV had significantly higher ($P<0.01$) placental tissue serotonin concentration. The mean (± SE) rectal temperaturewas significantly ($P<0.01$) increased on day 7 in group II and days 0 and 7 postpartum in group IV, from the study it was concluded that dose of 1800 CDU collagenase had significantly higher ($P<0.01$) concentrations of placental tissue hydroxyproline and placental tissue total protein release.

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