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A review of applications and scope of intrauterine proteolytic enzymes therapy for treatment of uterine infections in dairy animals

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Abstract
The target of one calf crop per year in dairy cattle and in 13-14 months in dairy buffaloes could be achieved only when an animal normally calves, completes uterine involution timely with initiation of post-partum ovarian activity. But, the postpartum uterine infections pose a great hurdle in the achievement of this target due to the economic losses owe to cost of treatment, disturbed calving to conception period and therefore the prolonged inter-calving interval. Various types of antimicrobials are used for the treatment of uterine infections in dairy animals which results in its withdrawal in milk and ultimately leading to arising of antimicrobial resistance in dairy animals as well as in human beings the ultimate consumers of dairy products. Therefore, research is on-going throughout the world to have alternative therapies to antimicrobials with aim to reduce the veterinarian cost as well as antimicrobial resistance. Many alternative therapies like immune-modulators, metritis vaccines, bacteriophage therapy, ozone therapy, interleukins etc. have been tried with variable success throughout the world. Intrauterine proteolytic enzymes therapy is one of the alternative therapies that our lab is mainly focussing for prevention and therapeutics of the various grades of uterine infections. This review shall focus on mechanism of action of proteolytic enzymes, their efficacy for prevention and therapeutics of uterine infections of different grades as well as the future perspectives related to this therapy.

Keywords: Dairy animals, uterine infections, alternative therapies, proteolytic enzymes, trypsin

1. Introduction

The economics of the dairy enterprises could be maintained by the production of regular calf crops per year in dairy cattle and in 13-14 months in dairy buffaloes so that maximum genetic potential in terms of future generation can be exploited. The most important factor responsible for the regular calf crops is the healthy postpartum uterus and puerperium period. The puerperium period includes the calving interval to complete uterine involution (Return to its normal size and position), endometrial regeneration, resumption of ovarian activity (ovarian rebound) and maintenance of healthy uterus without infections (Usually during the first 6 weeks postpartum) [31, 43]. Therefore, a healthy peripartum period which includes the timely uterine involution and ovarian rebound decides the future fertility of the postpartum animal [12]. Any disturbances during this peripartum period are likely to reduce the lifetime reproductive and productive efficiency by prolonging the calvig to conception and thereby, inter-calving interval.

The occurrence of uterine infections is a major limiting factor in the occurrence of normal puerperium in dairy animals even in normal calving while, the incidence and the severity of uterine infections are aggravated in conditions like dystocia, premature birth, abortions, hydropsy of fetal membranes, induced calving, twinning etc.[46, 47, 48, 13, 14, 26]. It has been observed that even in normal parturition 80-100% of cows will have bacterial contamination within the uterus. This bacterial load is usually eliminated during puerperium period but still 25-40% metritis, 20% endometritis and 25-40% subclinical endometritis cases were being reported [44]. Galvao *et al.* [19] reported a prevalence of 5 to >30% and 11 to >70% for clinical and sub-clinical endometritis after parturition, respectively.

The therapeutic management of different uterine infections is done by using various types of antimicrobial preparations via parentral as well as intrauterine route.

The inadvertent use of these antimicrobials leads to the development of antimicrobial resistance (AMR) in animal as well as the ultimate consumer of dairy products i.e human beings [40]. Therefore, scientists across the world are doing research on combating the AMR and one approach is the use of various non-antimicrobial preparations (alternatives to antimicrobial therapies). Similarly, many alternative therapies have been used for the prophylaxis and therapeutics of different grades of uterine infections by various scientists across the world [32]. But, the therapeutic use of proteolytic enzymes likes Trypsin, Chymotrypsin and Papain is a novel approach to treat the uterine infections. This review shall also be focussing on mechanism of action and effects of proteolytic enzymes along with future perspectives as a novel therapeutic management approach for uterine infections in dairy animals.

1.1 Types of uterine infections

A healthy uterine environment is one of the most important factors for the maintenance of excellent fertility in dairy animals. The reproductive efficiency of dairy animals has declined over the period and uterine infections are one of the major causes for this decline [55]. Metritis complex is the series of various pathological conditions of post-partum uterus that involves retention of fetal membranes (RFM), puerperal metritis, clinical endometritis, sub-clinical endometritis and pyometra [48] therefore, uterine infections can be classified into four types as puerperal metritis, clinical endometritis, subclinical/ cytological endometritis and pyometra [46, 47, 32]. Generally, uterine infections are classified according to the clinical presentation and degree of severity, but assessing the type of uterine infection is difficult due to lack of precision or definitions among research groups. Therefore, during the 15th International Congress on Animal Reproduction it was suggested that there should be clear definition of uterine disease that researchers could adopt. Even though it is not possible to categorize every affected animal but, these definitions are particularly used for research [21].

Puerperal metritis is most commonly observed within 10 days of parturition and is characterised by acute systemic illness with an enlarged uterus and a purulent watery red-brown to viscous off-white fetid odour uterine discharge, pyrexia, reduced milk yield, dullness, anorexia, increased heart rate and dehydration [47, 5, 32]. Main period of onset is from parturition and recovery of the sensitivity of the pituitary gland to Gonadotropin releasing hormone (GnRH) at10-12 days postpartum. It is one of the most important postpartum disorders causing heavy economic losses due to prolonged open days and intercalving intervals [4].

Clinical endometritis is characterised by presence of a purulent (>50% pus) or mucopurulent (approximately50% pus, 50% mucus) uterine discharge detectable in the vagina of cattle after 21 days and 26 days postpartum, respectively [47, 32].

Subclinical endometritis also known as cytological endometritis is defined as inflammation of uterine endometrium and is usually determined on the basis of polymorphonuclear cells (PMNs) count in uterine cytology samples. An animal with PMNs count more than 18%, 10% and 5% between 20-33, 34-47, >50 days postpartum is considered positive for subclinical endometritis [47, 32].

Pyometra is characterised by the distension of the uterus with closed cervix due to accumulation of purulent or mucopurulent material. It occurs due to presence of functional corpus luteum on ovary [47]. Before identifying the types of microorganisms present in uterus (before and after parturition), one should understand the term microbiome which refers to a group of commensal microorganisms that live and co-exist with other species peacefully, but any disturbances (dysbiosis) of these natural populations can have an detrimental effect on normal physiological functioning and therefore, the health of animal [27]. Earlier, it was believed that uterus is sterile during pregnancy and contaminated with non-specific bacterial microflora through other animals and environment post calving. However, now with the era of metagenomics it has been depicted that pre-calving uterus is not sterile [6, 38] and it harbours varieties of bacterial microflora [19].

1.2 Alternative therapies to mitigate detrimental impacts of uterine disease in animals

Indiscriminate use of antimicrobials leading to emergence of AMR in humans and animals causes increased incidence of morbidity and mortality which ultimately causing culling and economic losses to dairy industry [33]. AMR is a peril to human and animal health and therefore, World Health Organization issued several guidelines on the use of antimicrobial drugs [2]. The public health officials and researchers also tried to reduce the reckless use of antibiotics and shifted their focus towards developing alternatives therapies to reduce the judicious use of antimicrobials in animals with uterine diseases, respectively [24]. Recently, Lima [32] reviewed various alternative therapies used for the prophylaxis and therapeutics of uterine infections in dairy animals.

In brief, the alternative therapies to prevent and treat uterine infections includes use of *E. coli* lipopolysaccharide (LPS) [52], bacteriophages which inhibits *E. coli* growth [36], Botanical essential oils based on carvacrol having antioxidant, anti-inflammatory, and antibacterial properties [18], Mannose which acts as antagonist of *fimH* (virulence factor of *E. coli*) [36], Chitosan microparticles (CMs) which are chitin derivatives and have broad spectrum antimicrobial activity [10],Paraffin which improves immunomodulation[1], Ozone therapy which works by inactivation of microorganisms, anti-inflammatory and pain killer action [16], Probiotics likeLAB strain (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140) isolated from vagina which has a strong ability to produce H_2O_2 make acidic pH of tract [3,39]. Recently, the antibiotic induced drug resistance developed by several pathogenic microorganisms is countered by use of the nano-antibiotics. Nanoparticle-based carriers combat the bacterial resistance by providing the antibiotics with better biofilm penetration and less toxicity [15, 20]. Also, the prophylactic use of metritis vaccines against uterine infections have been tried by some workers [35, 17, 37] with variable success.

2. Intrauterine Proteolytic enzymes therapy

2.1 Types and origin of proteolytic enzymes

The proteolytic enzymes which are mainly used for intrauterine therapy are chymotrypsin, trypsin and papain. Out of these three, trypsin and chymotrypsin belong to endopeptidases class whereas papain is both endo- and exopeptidase [42]. Both trypsin and chymotrypsin are produced as inactive zymogen (proenzymes- trypsinogen) which is secreted in response to stimulation by cholecystokinin in the pancreatic juice and activated by enterokinase (also called enteropeptidase) present in the small intestine by proteolytic cleavage [41]. Enteropeptidase cleaves off a hexapeptide (6 amino acid sequence fragment) from N-terminalof trypsinogen to produce trypsin, the active enzyme. Trypsin is solely required for activation of other trypsinogen molecules (autocatalysis) and all other pancreatic proenzymes to produce the active form, namely chymotrypsin [42]. Out of these, papain is of plant origin and is usually produced as a crude, dried material by collecting the latex, unripe fruits and chyle from the the papaya tree (*Carica papaya*) and mountain papaya (*Vasconcellea cundinamarcensis*).The purified papain may be supplied as powder or as liquid [28, 30, 51] and belongs to both exo- and endopeptidase class [56]. The available market formulation of proteolytic enzymes powder are trypsin (Sigma-Aldrich Product code-048K7021), chymotrypsin (Sigma-Aldrich Product code-086K7695), and papain (Sigma-Aldrich Product code-76220) by Sigma-Aldrich.

2.2 Mechanism of action of proteolytic enzymes
Proteolytic enzymes are hydrolytic in nature (Hydrolases) that have the capacity to breakdown proteins and fats. Trypsin und Chymotrypsin are excreted into the duodenum along with the pancreatic juices, where they are responsible for splitting fats (as esterases) and proteins (as proteases). The effective optimum pH for them to function lays around 8 while, from pH 3 their effect is reversibly inhibited. Trypsin splits L-Arginine and L-Lysine building blocks. Chymotrypsin has the splitting specificity for the amino acid Tyrosine, Tryptophan and Phenylalanine [42, 9]. The proteolytic enzymes have direct and indirect immunomodulatory effect. Papain works directly as a cystein-protease, with similar mechanism of action as like bacterial cystein-proteases from gram-negative anaerobes, on the CD_{14} molecule of macrophages and monocytes and increases their level of efficacy as the initiator of the acute-phase-reaction. Papain is effective as an esterase and peptidase simultaneously and effects the splitting of the 20 amino acids mainly bonds of Arginine, Lysine and Phenylalanine. It is effective over a very wide pH range [54, 22].

2.3 How proteolytic enzymes work in uterine infection?
The proteolytic enzymes *viz*. trypsin, chymotrypsin and papain have fibrinolytic and proteolytic activity in inflamed tissue and are considered as biological scalpels. The presence of infection in the uterus makes the pH alkaline and the activity of these enzymes can be ensued. So, these enzymes act well in inflamed environment or alkaline pH. This results in to breakdown of the products of infection, damaged cells and tissues present in the lochia content. This effect of proteolytic enzymes is supposed to support the cellular defence mechanism [11, 23]. These enzymes also have immune-modulatory effect (increasing the movement of leucocytes) [50] and causes stimulation of the contractility of the smooth muscles [29, 11] which may favour involution [23]. *In vitro*, enzymes inhibit the growth behaviour and survival of microorganisms. The proteolytic enzymes in combination have more inclusive effect against microorganisms. Gram positive and gram-negative bacteria, yeasts, protothecals, surface structures and toxins containing proteins, lipids or combinations of both and are degraded through these enzymes. In this way these enzymes have direct effect on microorganisms. The changes in the membrane protein lead to abolition in growth or the death of the bacterium [29]. The proteolytic enzymes combination was first used for the treatment of mastitis in the form of intramammary preparation as non-antibiotic therapy since very long period [57, 29].

2.4 Proteolytic enzymes to treat endometritis
Drillich *et al*. [11] was the first to describe the use of proteolytic enzymes in chronic endometritic cattle and suggested it as an alternative to antibiotics treatment as this combination has fibrinolytic activity in inflammed tissue and supposed to support the uterine cellular defence mechanism and also inhibited the growth and survival of microorganisms. Honparkhe *et al*. [25] administered proteolytic enzymes in the form of mastivexym ointment intrauterine in repeat breeder buffaloes and achieved 86.7% cure rate and 60% pregnancy rate in subclinical endometritic buffaloes and concluded that the combination of proteolytic enzymes in the form of mastivexym ointment can be used for the treatment of subclinical endometritis as an alternative approach to antibiotic therapy in buffaloes.
Singh *et al*. [51] used single intrauterine infusion of proteolytic enzymes in form of Mastivexym ointment (trypsin 8 mg, chymotrypsin 8 mg, papain 4 mg) to treat the subclinical endometritic cattle and reported that intrauterine proteolytic therapy would enhance pregnancy rate (70% in treated animals as compared to 20% in control). Bhavna *et al*. [7] conducted a study to evaluate effect of intrauterine infusion of proteolytic enzymes on postpartum estrus and observed higher overall conception rate with lesser no. of artificial inseminations per conception and shortened calving to conception interval (CCI) treated Holstein Friesian cows. Overall, the administration of proteolytic enzymes improved postpartum reproductive performance with in cows’ early commencement of postpartum estrus with better estrual CVM characters which are indicative of improved uterine health and better fertility in crossbred dairy cows.
Singh *et al*. [50] studied effects of proteolytic enzymes intrauterine therapy on treatment of subclinical endometritis (SCE) and reproductive performance in postpartum water buffalo cows. In conclusion, results indicated a reduction in endometrial inflammation and non-pregnant days after proteolytic enzyme treatment in buffaloes with SCE. In this study treating the cows with SCE by intrauterine infusion of a combination of proteolytic enzymes reduced the median days to pregnancy by 27 days and improved the overall incidence of pregnancy by 26% when compared to untreated cows with SCE. It was observed that, there was a reduction in PMNs cell percentage in the endometrium of buffalo cows with SCE following treatment with enzymes which indicated the resolution of inflammation.

2.5 Use of intrauterine proteolytic enzymes therapy in dystocia affected cases
Gulia *et al*. [23] conducted a study to analyse the intrauterine efficacy of combination of proteolytic enzymes (trypsin 16 mg, chymotrypsin 16 mg, papain 8 mg) in dystocia affected buffaloes and reported an early expulsion of fetal membranes (8.80±1.50 h *vs* 27.40±6.33 h) with reduced chances of retained fetal membranes (26.67% *vs* 73.33%), decreased systemic illness as indicated by reduction in neutrophils count (55.05±1.63 *vs* 64.92±1.46% on day 45 postpartum), earlier uterine involution in treated animals with delayed involution in 73.33% animals of control group, lesser percentage of overall postpartum uterine infections (6.67% *vs* 53.33% on day 45 postpartum).The study also reported that there was greater milk production (10.98±0.80 *vs* 7.65±0.91 Kg average daily peak milk yield) with 85% achievement in peak milk yield of previous gestation as compared to only 50% achieved in control with earlier onset of first postpartum estrus in treated animals (44.80±30.6 *vs* 54.15±3.89 days), lesser

number of artificial inseminations required per conception (3.16 *vs* 5.33) and higher overall pregnancy rate (40% *vs* 20%) with lesser number of animals becoming anestrus (26.67 *vs* 66.67%) in treated than control group.

2.6 Effect of proteolytic enzymes on Interleukins and Acute phase protein levels

Singh *et al*. [51] conducted a study to evaluate effects of intrauterine administration of proteolytic enzymes on endometrial interleukins levels in buffaloes with SCE. Uterine concentrations of IL-1β were reduced, whereas IL-6 was increased following enzymatic treatment. Cows diagnosed with SCE on day 21 postpartum had more ($p<0.05$) concentrations of IL-8 and TNF-α in serum as well as uterine flushing samples, while IL-1β concentrations were more in the uterine flushing samples only; when compared to cows without SCE. On day 28 postpartum, all cows with SCE had greater concentrations of IL-1β and TNF-α in both serum and uterine flushing samples (IL-8 only in uterine flushing samples) compared to cows without SCE.

Bisla *et al*. [8] analysed serum concentration of Fibrinogen, Haptaglobin, Serum Amyloid A (SAA), Acid-1 glycoprotein (AGP), Lactoferrin, Interleukin-6 and 10 in dystocia affected animals on day 20 and 45 post-partum. The concentration of Haptaglobin and Fibrinogen was lesser on day45 ($p<0.05$) in animals treated with enzymes therapy than control while, the concentration of both increased on day20 and 45 than day0. SAA, lactoferrin and AGP varied non-significantly ($p>0.05$) between control and treatment groups on both day20 and 45 post-partum. Interleukin-6 and 10 concentrations were significantly higher ($p<0.05$) on day20 as well as 45 in control group than treatment. The concentration of Fibrinogen, Haptaglobin, SAA, Interleukin-6 and 10 were significantly higher ($p<0.05$) in the animals having uterine infections on both day 20 and 45 irrespective of treatment.

3. Future Prospective

The proteolytic enzymes due to their fibrinolytic and proteolytic activity in inflamed tissue with immuno-modulatory effect (increasing the movement of leucocytes), support the cellular defence mechanism and have direct effect on microorganisms leading to stasis in their growth or cause their death. All these properties of proteolytic enzymes make them a potential candidate to be used for uterine infections treatment without developing any resistance as developed with the use of antimicrobials. So, use of these enzymes can replace the use of antimicrobials and acts as an alternative therapy used for the prophylaxis and therapeutics of uterine infections. But, more research in larger population is warranted to establish the effect of the therapy. There is also need to establish the market preparations of these enzymes for their utility as therapeutics of uterine infections which need *in-vitro* as well as *in-vivo* trails.

4. Conclusion

It can be concluded that the use of intrauterine proteolytic enzymes therapy reduces the chances of development of uterine infections by causing earlier expulsion of placenta, reduced neutrophils count, faster uterine involution, earlier resumption of ovarian activity, increasing postpartum reproductive efficiency in terms of lesser days from parturition to first estrus, lesser number of artificial inseminations required per conception, higher milk yield and overall pregnancy rates. As well, it is a good therapeutic regimen for the treatment of clinical and subclinical endometritis in dairy animals and can be used in routine applications.

5. Conflict of Interest

The authors have no conflict of interest to declare among themselves.

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