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# THE IMPACT OF INTRACERVICAL HYALURONIDASE ENZYME ON CERVICAL HISTOMORPHOLOGY IN SUCCESSFULLY DETORTED UTERINE TORSION AFFECTED BUFFALOES

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## ABSTRACT

The present study evaluated the impact of intracervical hyaluronidase enzyme on cervical histomorphology in successfully detorted uterine torsion affected buffaloes. These animals were either subjected to routine post-detorsion treatment (n=10) or in addition to routine treatment, hyaluronidase enzyme (10,000 IU; 2.5 ml at each of 3, 6, 9, 12 o'clock position of cervix) was administered intracervically immediately post-detorsion (n=10), followed by repeated administration at 6 h interval (0, 6, 12, 18 h) till complete cervical dilatation or till 24 h after detorsion. The pre-treatment cervical biopsy samples were collected immediately after detorsion and post-treatment samples were collected at complete cervical dilatation which happened within 24 h after detorsion in all the cases. Following histomorphology, the quantification of collagen fibers as mean percentage area occupied collagen out of total tissue area in pre-treatment cervical biopsy samples of all the buffaloes was revealed as 21.0±5.7. During post-treatment period, at the time of complete cervical dilatation, this value exhibited a decrease in control (9.1±2.2; $P>0.05$) as well as hyaluronidase group (5.8±2.1; $P<0.05$). In summary, collagen fiber dispersion in cervical tissue of successfully detorted uterine torsion affected buffaloes suggested their role in cervical dilatation, whereby, intracervical hyaluronidase was able to enhance the cervical tissue collagen dispersion.

**Keywords**: *Bubalus bubalis*, buffaloes, cervix, histomorphology, hyaluronidase, uterine torsion

## INTRODUCTION

The torsion of uterus, a more frequent maternal cause of dystocia in buffaloes compared to cattle, usually occurs in a pregnant horn and is defined as twisting of

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uterus on its longitudinal axis (Purohit *et al*., 2012). Following successful detorsion of uterus, around 18 to 50% buffalo fail to exhibit complete dilatation of cervix (Ghuman, 2010). Subsequent to detorsion procedures, the presence of dead fetus may lead to hardening of cervix within 24 h, if immediate cervical dilatation approaches are not practiced (Honparkhe *et al*., 2009).

In the beginning of parturition, there is a reduction in the concentrations of collagen and other glycosaminoglycans concurrently with an increase in the concentration of hyaluronic acid. The latter is found in high concentration prior to an increase of prostaglandin $E_2$, relaxin and progesterone antagonist (Kobayashi and Terao, 1997). Further, at the time of fetal delivery, Interleukin-1α and hyaluronic acid could release hyaluronidase in the cervix (Kobayashi and Terao, 1997). Hyaluronidase is a local action enzyme which is known to reduce the cellular adhesions in cervix by depolymerization of cervical conjunctive components (collagen, hyaluronic acid and chondroitin), thus causing cervical softening and dilation (Li *et al*., 1994). Hyaluronidase causes hydrolysis of hyaluronic acid by breaking glycosamidic link between the C1 of glycosamine and C4 of glycuronic acid (Obara *et al*., 2001). Intracervical hyaluronidase (10,000 IU) in partially dilated cervix of uterine torsion affected buffaloes lead to complete dilatation in 87.5% buffaloes (Singh, 2018). The present study in uterine torsion affected buffaloes aimed to assess the impact of intracervical hyaluronidase enzyme on cervical histomorphology related to cervical dilatation.

## MATERIALS AND METHODS

### History and obstetrical treatment

The present study was carried out on twenty full term pregnant buffaloes presented for the treatment of uterine torsion at Teaching Veterinary Clinical Complex, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. The history of each uterine torsion affected buffalo with regard to parity (80% in 2$^{nd}$ to 4$^{th}$ parity) and duration of occurrence of uterine torsion (50%: <18 h; 50%: 18 to 36 h) as judged from milk resorption and sacrosciatic ligament relaxation was recorded. All the presented buffaloes had right-side post-cervical uterine torsion and 65% of these buffaloes had ≥180° torsion. Subjecting all the buffaloes to Sharma's modified Schaffer's method of uterine detorsion lead to successful uterine detorsion either in one (35% buffaloes), two (55%) or ≥ three rolls (10%). Following successful uterine detorsion, the cervical status in the buffaloes was observed as either closed or partially dilated (<4 fingers or up to 7 cm diameter open).

### Groups

Based upon the history and obstetrical observations, all the buffaloes were equally divided into control and hyaluronidase Treatment groups. Immediately after detorsion, the Control group buffaloes, through per-vaginal route, were subjected to intracervical administration of 0.1 M sodium phosphate buffer using 21G scalp vein (2.5 ml at each of 3, 6, 9, 12 o'clock position of cervix) at 6 h interval until complete dilatation of cervix or till 24 h post-detorsion. In hyaluronidase group, intracervical hyaluronidase [10,000 IU: 20 mg hyaluronidase powder (500 IU/mg MP biomedicals Australasia Pvt. Limited, Australia) dissolved in 10 ml 0.1 M sodium phosphate buffer] treatment

was given as per the procedure adopted for control group. In addition, immediately after detorsion, all the buffaloes of both the groups were administered (i.m.) 500 µg cloprostenol sodium and 40 mg dexamethasone along with the routine supportive therapy.

**Collection of cervical tissue samples**

The buffaloes were restrained using low epidural injection (3 to 5 ml, 2% lignocaine hydrochloride) followed by cervical tissue sample collection from dorsal side of cervix about 2 cm inside the external orifice. The first sample was collected immediately following uterine detorsion, before administering intracervical hyaluronidase or sodium phosphate buffer, using a 6 mm diameter biopsy punch. The second sample was collected at complete cervical dilatation which happened within 24 h post-detorsion in all the cases. Immediately after biopsy collection, the samples were fixed in 10% neutral buffered formalin for 48 h.

**Histomorphology procedures**

Cervical tissue samples were washed in running tap water overnight to remove excess fixative followed by exposure of samples to ascending grades of alcohol (70%, 80%, 90% and absolute alcohol) and acetone for dehydration and benzene for clearing. Thereafter, the samples were infiltrated and embedded in paraffin wax. The sections of 4 to 5 µm thickness were subjected to Haematoxylin and Eosin (H&E) stain for tissue morphology demonstration (Luna, 1968) and Picrosirius Red (PSR) stain for collagen fibers (Choudhary *et al*., 2017). For staining of collagen fibers, 5 µm thick sections were deparaffinized with xylene and hydrated to water (using descending grades of alcohol). Nuclear staining was done using Weigert's hematoxylin for 7 minutes, washing in running tap water for 10 minutes followed by 0.1% PSR in the saturated picric acid solution for 60 minutes. Stained slides were rinsed twice in acidified water. Finally, sections were dehydrated in ascending grades of alcohol, cleared in xylene and mounted with DPX.

**Quantification of staining intensity**

ImageJ (ver 1.51f) software was used to count PSR stained area of biopsy samples (Choudhary *et al*., 2017). The stained slides were imaged using Nikon microscope (80i) with photographic unit. Ten representative photomicrographs (at 400X) were captured in .tiff format from each biopsy tissue (one tissue/animal). Thereafter, PSR stained red areas of collagen were selected by placing markers of different colors to generate the immune histochemistry index. The pixel intensity of the images was calibrated with scale bar, thus, the percent collagen area occupied out of total area of tissue was calculated. The results were saved individually for each animal, separately for pre- and post-treatment. The data was pooled to each category of treatment and was analyzed statistically.

**Observations**

Cervical histomorphological variations, collagen distribution pattern and polymorph nuclear (PMN) cells were recorded and compared in both groups. The H&E stained images were evaluated for epithelium, propria submucosa, blood vessels (hyperemia and hemorrhage) and infiltrating cells. The 400x images were used to calculate cervical tissue collagen fibers per unit area (mean value of percentage of area occupied out of total area of the tissue).

**Statistical analysis**

The data of pre-treatment cervical tissue collagen fibers per unit area was pooled for buffaloes of both the groups and was compared with post-treatment values of control and hyaluronidase group using *t*-test (SPSS, version 16.0). The data was presented as mean±SE and minimum significant interaction was considered at 5% level.

## RESULTS AND DISCUSSIONS

**Histomorphology of cervical tissue collected from non-dilated cervix immediately after detorsion**

Cervical histomorphology revealed normal cervical histoarchitecture without heavy vascularization and multiple infiltrations of PMN cells. In brief, the cervical mucosa had mucosal folds with primary and secondary branches. The lining epithelium was columnar type with pseudostratified epithelium at some places. The columnar cells had elongated nuclei located towards basement membrane. The apical surface of these cells had either cilia or secretary blabs. The goblet cells were observed at places in the lining epithelium. The propria submucosa had loosely arranged connective tissue fibers with capillaries and few infiltrating cells (neutrophils, eosinophils and lymphocytes; Figure 1 and 2).

Further, the collagen fibers appeared red and nuclei appeared grey/brown with elongated cells of collagen fibers arranged in parallel rows. The infiltration of PMN cells was observed in the propria submucosa. The qualitative assessments showed collagen fibers as closely arranged with little inter-fiber spaces. Blood vessels had slight congestion with less frequent hemorrhages (Figure 3 and 4). The successful delivery of a fetus through complete cervical dilatation due to separation of collagen fibers and imbibition of fluids in the cervical tissue were the primary requirements (Winkler *et al*., 1999; Uchiyama *et al*., 2005). The quantification of collagen fibers in cervical sections collected immediately after detorsion of buffalo revealed cervical tissue collagen fibers per unit area as 21.0±5.7. In previous studies, the higher density of collagen fibers per unit area was suggested in a non-dilated cervix of uterine torsion affected buffaloes (Singh, 2018).

**Histomorphology of cervical tissue at complete cervical dilatation**

In control and Hyaluronidase group buffaloes, the epithelium was lined by pseudostratified and columnar cells. The lamina propria consisted of loosely arranged collagen fibers. Inter-collagen space was filled by homogenous/watery substance. As compared to pre-treatment biopsies, the blood vessels had RBC's along with abundant PMN cells in between the loosely arranged collagen fibers (Figure 5 to 8). The accumulation of water in interstium between the collagen may promote dispersion or prevent aggregation of collagen fibrils, thus weakening the tensile strength of the matrix (El-Maradny *et al*., 1997). Hyaluronidase play important role by promoting cervical relaxation either directly via tissue hydration by attracting water molecules or indirectly via regulation of inflammatory genes (Dowthwaite *et al*., 1999; Uchiyama *et al*., 2005). In fact, there was an extensive increase in the PMN cells in the Hyaluronidase group (Figure 7). The heavy vascularization and multiple infiltrations of PMN cells in cervical tissue are one of the pre-requisites for the cervical dilatation (Nagase and Woessner, 1999). Furthermore, compared to pre-treatment value (21.0±5.7), the cervical tissue

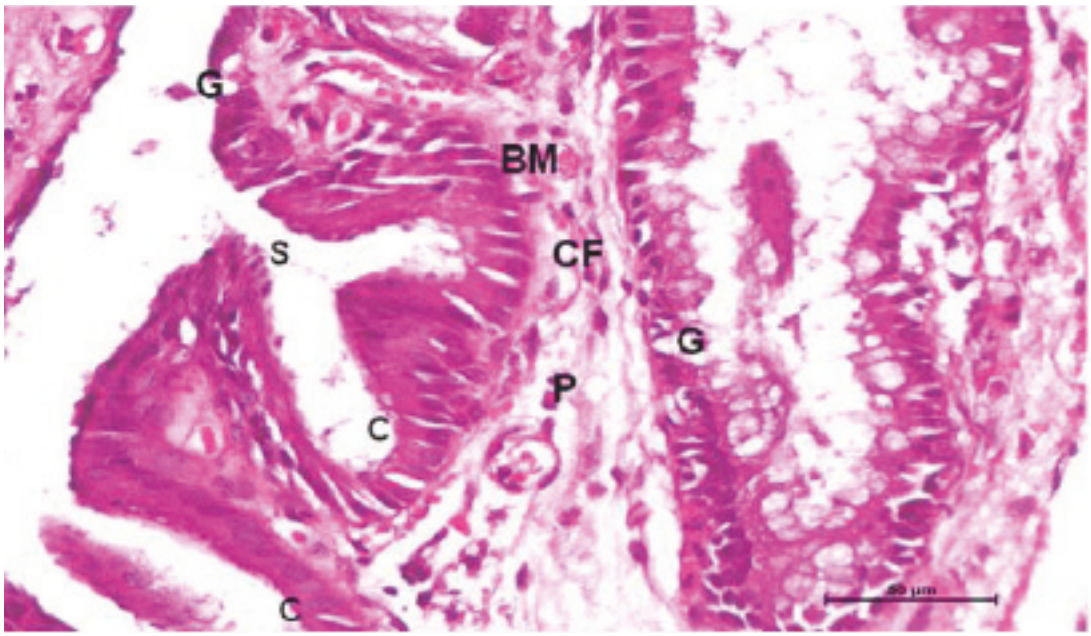


Figure 1. Pre-treatment, Non-dilated cervix: Section (H&E, 400X) showing laminar epithelium consisting of columnar epithelium over basement membrane (BM). Lamina epithelia contained ciliated cells (C), secretory (S) and goblet cells (G). *Propria submucosa* contained collagen fibers (CF) and polymorphonuclear cells (P).

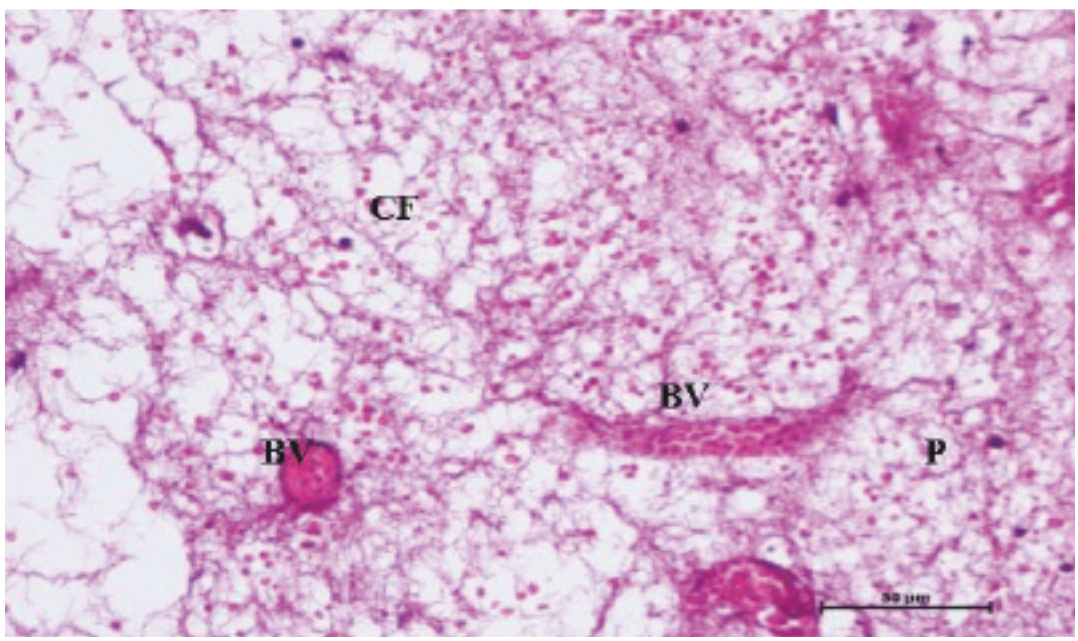


Figure 2. Pre-treatment, Non-dilated cervix: Section (H&E, 400X) showing Blood vessels (BV), collagen fibers (CF) and polymorphonuclear cells (P).

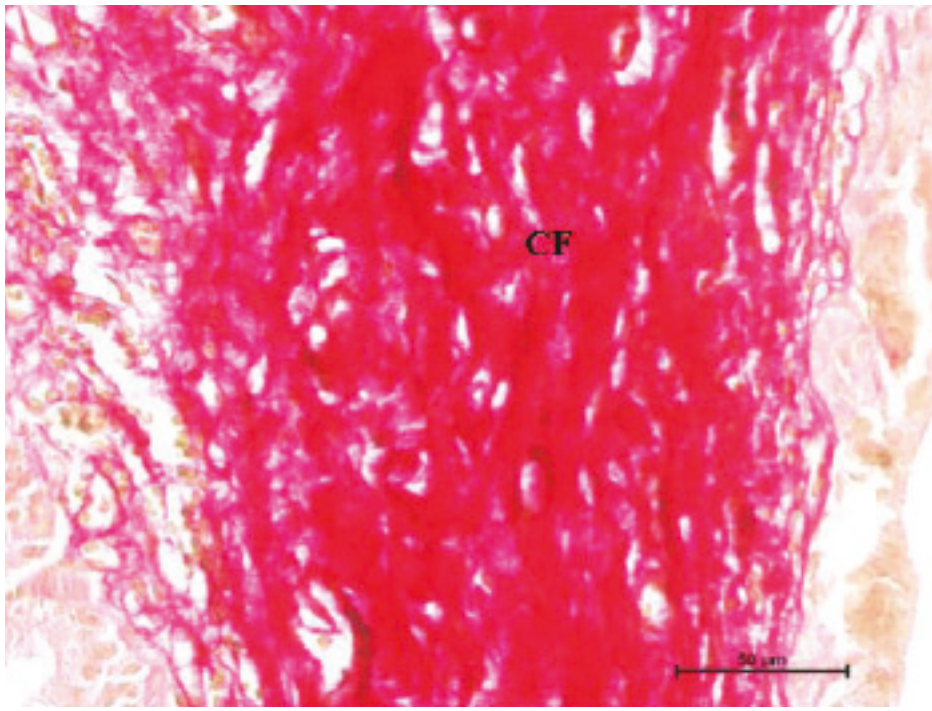


Figure 3. Pre-treatment, Non-dilated cervix: Section (PSR, 400X) showing cervical epithelium lined with columnar cells (C), propria submucosa with closely placed and darkly stained collagen fibers (CF) and few goblet cells (G).

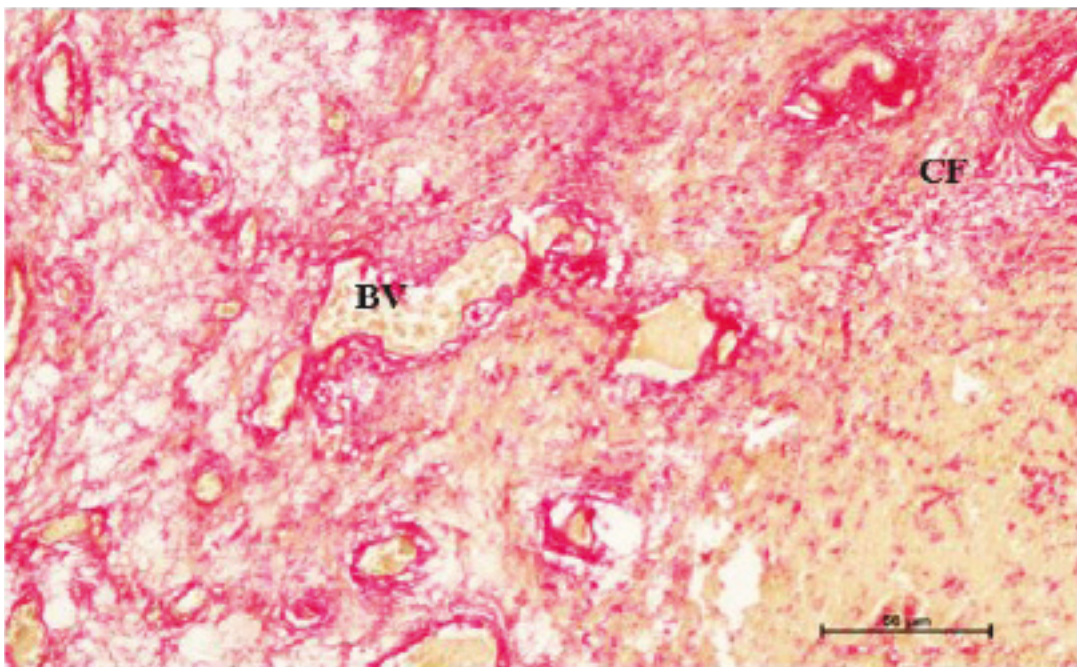


Figure 4. Pre-treatment, Non-dilated cervix: Section (PSR, 400X) showing propria submucosa with closely placed and darkly stained collagen fibers (CF) and blood vessels (BV).

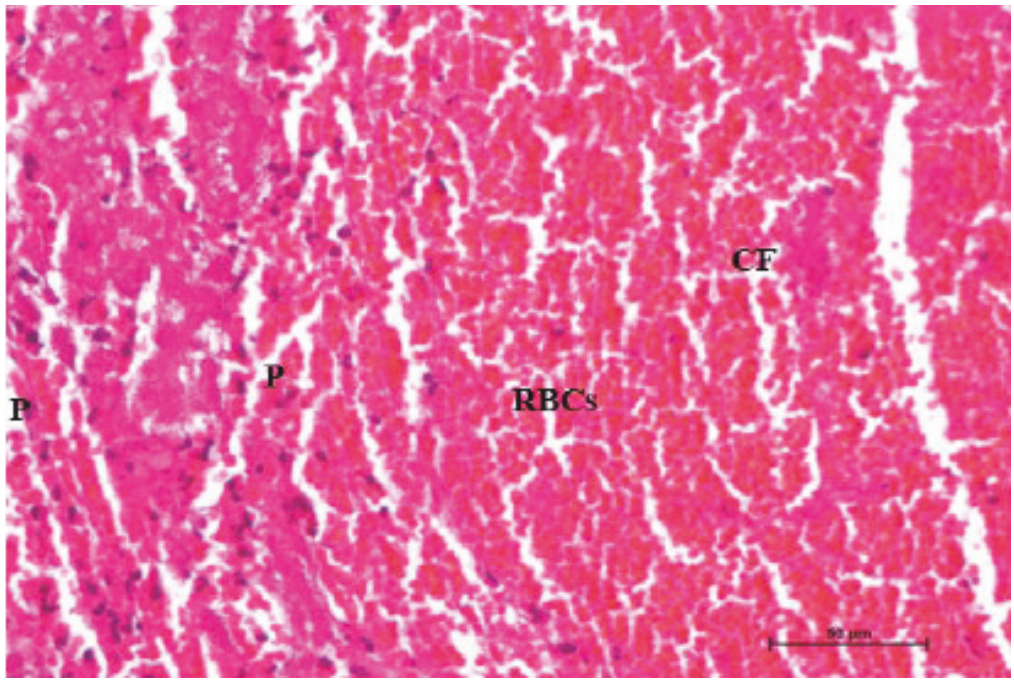


Figure 5. Control, Post-treatment, Dilated cervix: Section (H&E, 400X) showing haemorrhage (RBCs), collagen fibers and infiltration of polymorphonuclear cells (P).

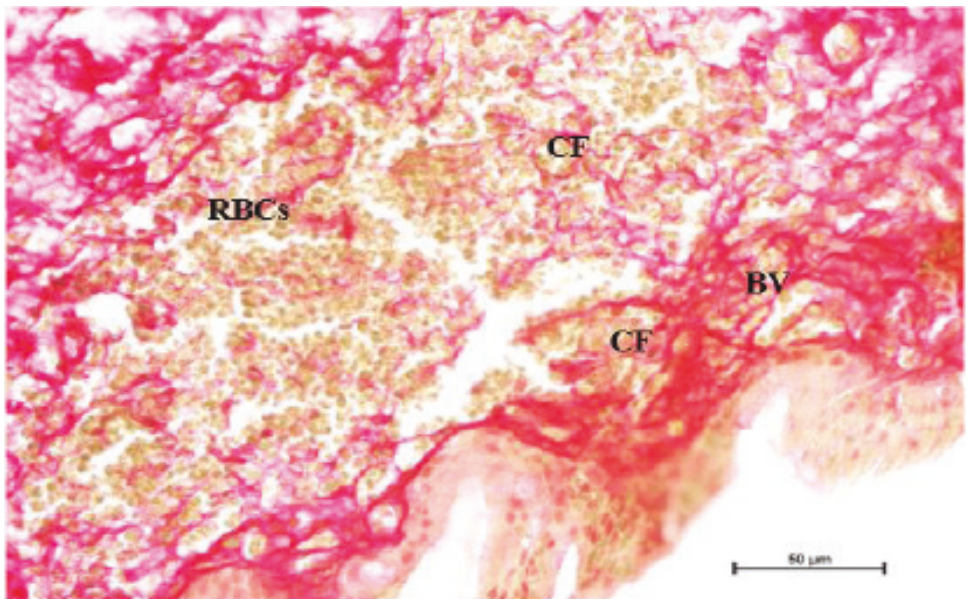


Figure 6. Control, Post-treatment, Dilated cervix: Section (PSR, 400X) showing propria submucosa with distantly placed collagen fibers (CF), haemorrhages (RBCs) and congested blood vessels (BV).

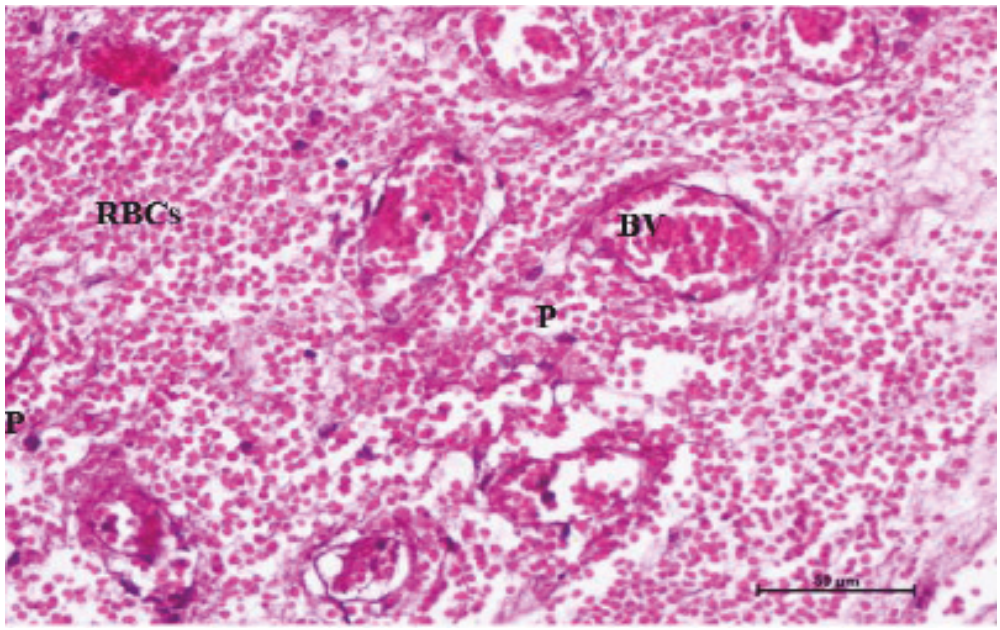


Figure 7. Hyaluronidase, Post-treatment, Dilated cervix: Section (H&E, 400X) showing engorged blood vessels (BV) with extensive haemorrhage and polymorphonuclear cells (P).

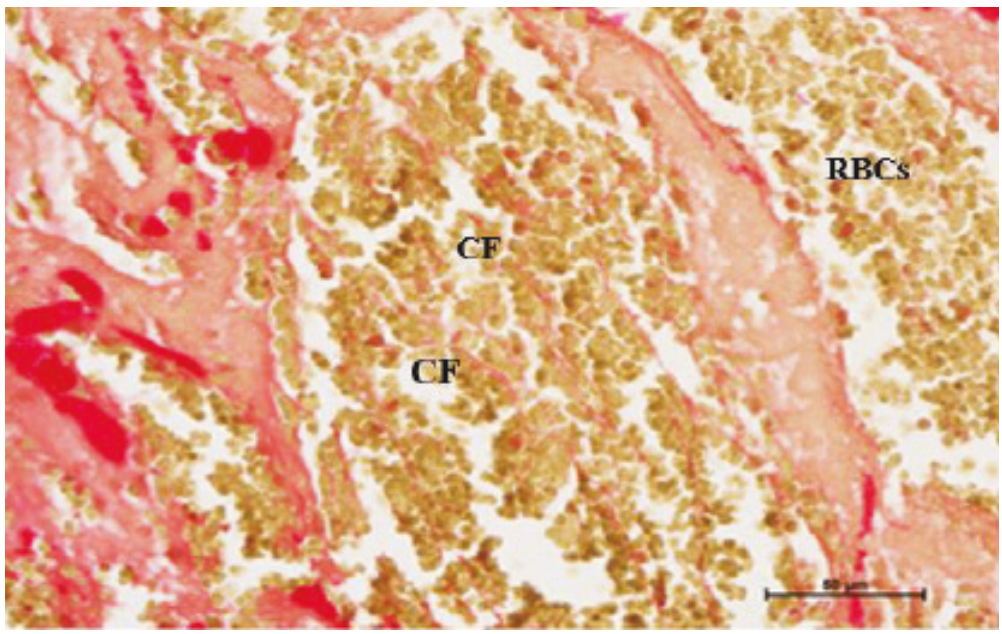


Figure 8. Hyaluronidase, Post-treatment, Dilated cervix: Section (PSR, 400X) showing propria submucosa with distantly placed collagen fibers (CF), haemorrhages (RBCs) and congested blood vessels.

collagen fibers per unit area at complete cervical dilatation was reduced to 9.1±2.2 (P>0.05) and 5.8±2.1 (P<0.05) in control and Hyaluronidase group buffaloes, respectively. This decrease in the area fraction of collagen fibers might have led to complete cervical dilatation.

In conclusion, the histomorphological data from cervical tissue samples collected immediately after detorsion and at complete cervical dilatation suggested that hyaluronidase enzyme may have a role in collagen dispersion and influx of PMN cells, which play a role in cervical dilatation, but these were not the only factor responsible for cervical dilatation.

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