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STANDARD PROTOCOL FOR INTESTINAL CANNULATION OF RUMINANTS

Juraj SZAKÁCS*, Ľubica CHRASTINOVÁ, Mária CHRENKOVÁ

NPPC – Research Institute for Animal Production Nitra, Lužianky, Slovak Republic

ABSTRACT

The manuscript describes the technique for duodenal cannulation of beef cattle using our modification of the standardized closed T-cannula (manufactured by Bar Diamond, Inc., USA). This technique is utilized to determine intestinal digestibility (mobile bag) and, in combination with rumen cannula, also the total digestibility of experimental feeds using the method *in situ.* We describe the main principles of pre-operative preparation of the experimental animals as well as post-operative care, in order to ensure problem-free and long-term utilization of the animals in experiments. We offer a brief overview of the development of surgical techniques and methods used to determine feed digestibility. Development of these methods is clearly progressing towards minimal invasiveness for the experimental animals. Russel's principle of 3 Rs (Replacement, Reduction, Refinement), and expanded by Tannenbaum and Bennet in 2015, is today applied not only due to the progressively stricter European legislative, which the Member States are obligated to implement into their regulations, but from the practical standpoint as well (Russell, 1959). These methods allow saving labour and material costs and the time. The aim of this study was to describe standard protocol for intestinal cannulation of ruminants.

Key words: surgery; cannulation; duodenum; ruminants; pre- and post-surgery care; mobile bag method

INTRODUCTION

Cannulated ruminants, which enable the use of the mobile bag method (Figure 2), are necessary to determine predicted intestine-enzymatic or total digestibility of feed after 24 h incubation *in situ* in the rumen (Chrenková *et al.*, 2012; 2018). Usually, the cannula is firm and comprises a fixation base and a tube (patent Bar Diamond Inc., USA). For various categories and sizes of animals, as well as for various sections of the intestine, a wide range of cannulas are offered. The cannulas allow, in addition to insertion of sealed bags with tested feed, also intermittent collection of chyme.

The aim of this study was to describe standard protocol for intestinal cannulation of ruminants.

MATERIALS AND METHODS

Duodenal cannula

For the surgery, a T-cannula, manufactured by Bar Diamond, Inc. from the USA, was used. The description and composition is presented on the website https://shop.bardiamond.com/en/ic1-o-intestinal-cannula-open-12. Cannula was modified by paring the inner, peritoneal disk (Figure 1).

Preparation of animals

Animals were selected according to the methodical aim, in order to utilize them for a long-term period. Most suitable were young heifers with only a small amount of subdermal and depot fat, making the surgery significantly easier. We used in

***Correspondence:** E-mail: juraj.szakacs@nppc.sk,
Juraj Szakács, NPPC – Research Institute for Animal Production Nitra,
Hlohovecká 2, 951 41 Lužianky, Slovak Republic
Tel.: +421 376546229

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this study Holstein Fresian cattle (1.5 years old, 250 kg). In the first phase, it was necessary to acclimate the animals to tied-down housing as well as daily physical contact in the area of the hungry fossa. This daily "training", connected to feeding, cleaning and washing of the animals, continues after the surgery with a pause after the removal of stitches. The experimental animals, thus, do not develop negative association related to the short-term pain after the surgery, which later eliminates the necessity to sedate and fixate them during manipulation with the cannula, when samples are taken in experiments. In case of intestinal cannulation, pre-surgical diet is necessary (hay for 48 hours) as well as elimination of the evening feeding the day prior to the surgery. Water is made unavailable the morning of the surgery.

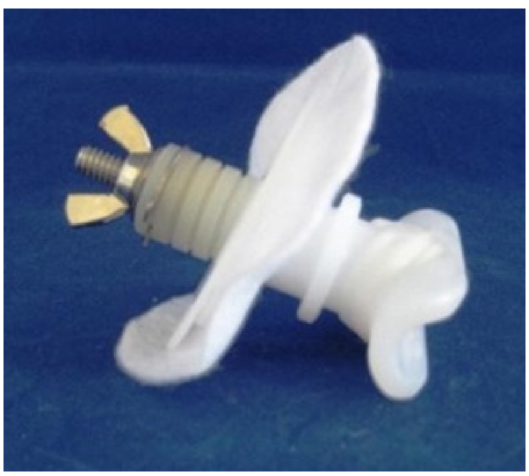
Longer fasting can lead to changes, due to sagging of the rumen in stomach topography and makes access to proximal duodenum worse or even impossible.

The surgery itself comprises several phases:

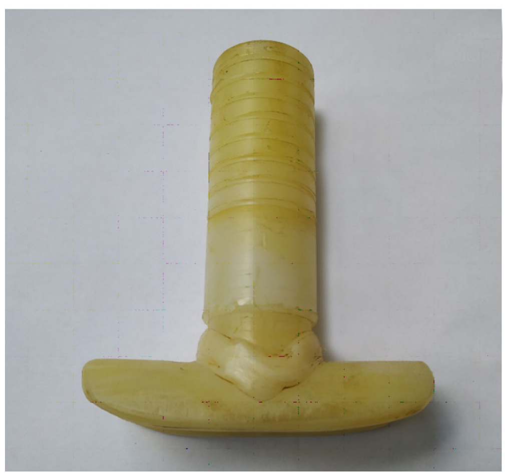
Preparation of the surgical site (after shaving the area of the hungry fossa the day prior) consists of mechanic cleaning, followed by degreasing of the surgical site (Benzinalcohol) and then disinfection using Ajatin tincture. Similarly prepared is the cannula, which is the night before the surgery put into clean water with Ajatin solution.

Sedation and anaesthesia. Sedation using xylazine (Rometar 2 % inj.) in amount 0.10 – 0.15 ml.100 kg^{-1} live weight. In addition to its analgesic and myorelaxation effects, xylazine is also characterised by its ability to act on the digestive tract's motor functions. Therefore, we recommend the use of minimal dose necessary to calm and immobilize the animal. Overdose can lead not only to the animal reclining, but it can cause long-term proventricular atony. Therefore, we focus on quality local anaesthesia using 2 % solution of procaine (Procain SPOFA 10 % A.U.V. inj.50 ml, with active substance Procaini hydrochloridum) at maximum dose 4 $mg.kg^{-1}$ l.w. starting with subdermal layer, through all muscle layers along the expected incision. When peritoneum is uncovered, a few drops can be applied to its surface as well. Procain is applied to the site of the cannula's exit as well, but only after its fixation in the intestine. Sufficient local anaesthesia is achieved fifteen minutes from application. Before the beginning of the surgery, antibiotics Norostrep inj. or Shotapen inj. are also applied at the recommended dose.

Resection of stomach wall in length 10 – 12 cm in the right stomach cavity in slightly cranioventral direction was made in order skin (*cutis*), hypodermis (*subcutis*) and muscle layers (*m. cutaenus trunci, m. obliquus externus et internus abdominis, m. transversus abdominis*). The veins of each layer are immediately ligated to prevent bleeding into the



A: Original



B: Modified

Figure 1. Duodenal cannula

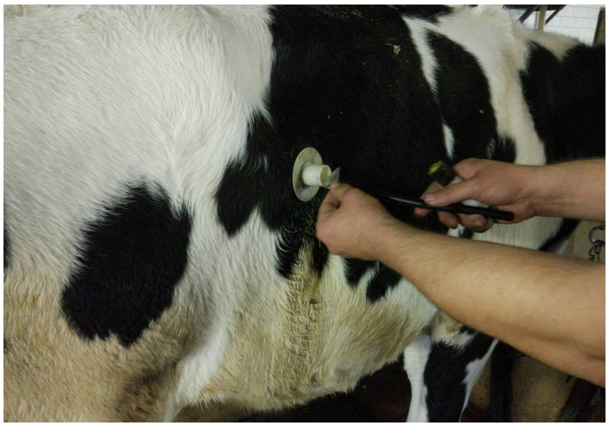


Figure 2. Insertion of mobile bags into duodenum

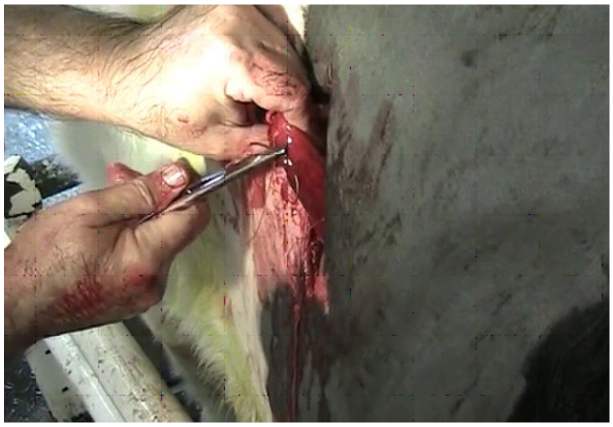


Figure 3. Placing doubled circular sutures to the isolated section of duodenum

stomach cavity when the peritoneum is cut. Slightly pulled out peritoneum, fixed with forceps, and is cut under the control of hand using mayo scissors, never in the length of the entire surgical wound. It is sufficient if the surgeon's hand can enter the stomach cavity.

Fixation of the proximal duodenum using sterile hydrophilic gauze bandage, which is inserted from the caudal side through the opening in *omentum majus*. Caution is necessary around the veins in *omentum*, but the same is true naturally also when selecting the section of duodenum with minimum of larger veins, in order to prevent massive hematoma, which would make the site of cannulation difficult to navigate.

Fixation sutures. On the isolated *duodenum* on free surface without *Omenta majus*, two oval circular seromuscular sutures are made using Silon A4 (Figure 3). Outer should be 3 mm from the inner suture, to preserve the surface as well as mucous membrane. Length approximately 5 cm, width – 4-5 mm. Each suture is started from the opposite end.

Insertion of the cannula. While the sutures are prepared, the cannula is placed into warm water with Ajatine. The section of the *duodenum* with circular sutures is gently pulled outside the stomach cavity, using fixation bandage. In the centre of the inner suture, the epicardium, mucosa and submucosa are cut with care to avoid cutting through the sutures. The mucous membrane is cut using mayo scissors or bowel scissors. The intestine is emptied and cleaned of content. Base of the cannula is inserted into the opening; the opposite end is inserted into the opposite end of the intestinal incision with care to avoid damaging the submucosa and tearing of the inner suture (Figure 4). After ensuring that both ends of the fixation base are in the *luminum* of the *intestine*, the inner suture is immediately firmly tied off. The surface of the intestine as well as the cannula's tube are thoroughly cleaned by wiping them with sterile bandage outside the stomach cavity and washed with warm physiological solution. The cleaned tube is closed using a bandage tampon. The outer suture is tied off in such a way as to intussuscept the remnants of the mucous membrane inside. The closed intestine with cannula is examined for permeability from both ends using Janeta with

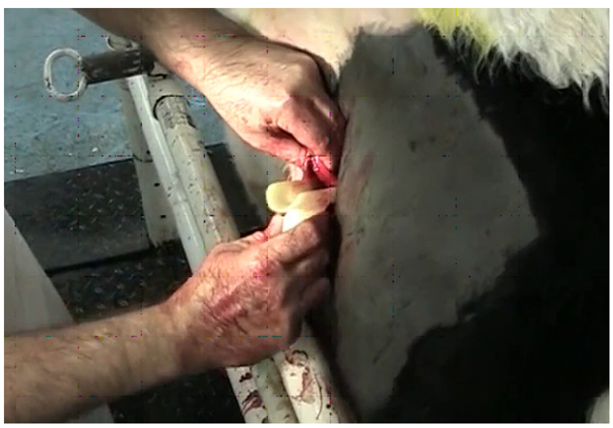


Figure 4. Insertion of cannula into the intestine

round-end urinal catheter (Figure 5). After warm physiological solution is applied, the cleaned tube is again closed using a tampon and reinserted into the stomach cavity.

Guide the cannula to the surface of the body. The cannula is always guided out outside the main incision. This will prevent it from coming loose and speed up the healing of the incision. Approximately 3 cm outside the incision, after an injection of 5 ml 2 % Procaine, cut 3 – 4 cm of the abdominal wall in layers. Pierce the peritoneum with scissors under the control of fingers, expand manually from the inside. In the case of bleeding, short-term bilateral compression of the incision using fingers is usually sufficient. Only in exceptional cases is it necessary to use a suture. Additional compression is provided by a pulled-out and firmly fixed tube. Great care must be taken not to pull the peritoneum out of the main incision when pulling out the cannula, thus preventing the abdominal cavity from sealing. Grasp the cannula tube. An assistant inserts three long forceps into the incision under control from the inside. The surgeon applies these alongside the tube. The tube is pulled out of the wound by hand with simultaneous manual fixation and pressure of the intestine towards the abdominal wall (Figure 6). Fix it firmly using a hydrophilic bandage. The permeability of the intestine is examined again.

Closure of the abdominal cavity. Close the peritoneum and fascia with A4 nylon lock-stitch sutures. The second layer using the same material again with lock-stitch sutures, and the third the same way. Be careful not to create pockets. Skin with subcutaneous tissue is sutured using Silk A6 lock-stitch sutures or U sutures. Although this method noticeably prolongs the procedure, it is necessary to prevent tearing of the sutures and forming of pockets in the event of high intra-abdominal pressure and swelling. Cover the closed main incision with antibiotic ointment (Framykoin ung.).

Complete the cannula on the skin by fixing the tube with a plastic pad and a flexible metal sleeve. Spread this fixing sleeve with the help of pliers and slide it through the tube up to the last groove (Figure 7). Close the tube using a stopper (Figure 8). Wipe the site of the cannulation with Septonex or coat it using Framykoin ung.

After the surgery is completed, a dose of analgesics (Novasul inj., Richterpharma AG) is applied to prevent the animal from ripping out the cannula by defensive reactions when the effect of the local anaesthesia ceases. Analgesics are applied as needed for several days after the surgery, which decreases the post-operative stress and shortens the time of healing of the wounds as well as prevents defensive reactions of the animals during manipulation.

A very important aspect is to ensure suitable housing of the cannulated animals, in order to prevent tearing out the cannulas when the animals lie down or stand up.

In long-term use of the experimental animals, vitamins (ADE-Vit a.i.v, inj., Bioveta, Ivanovice na

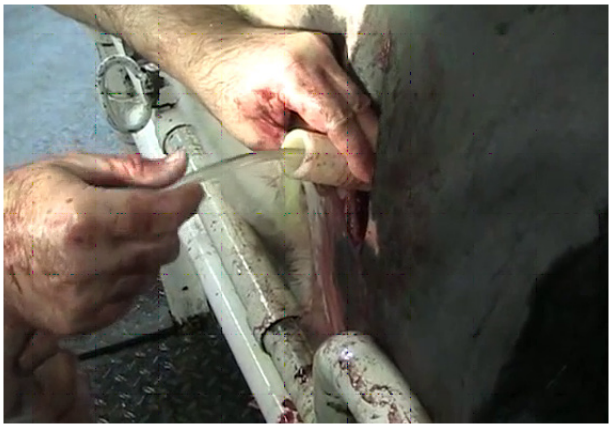


Figure 5. Control of duodenum's permeability after cannula's fixation

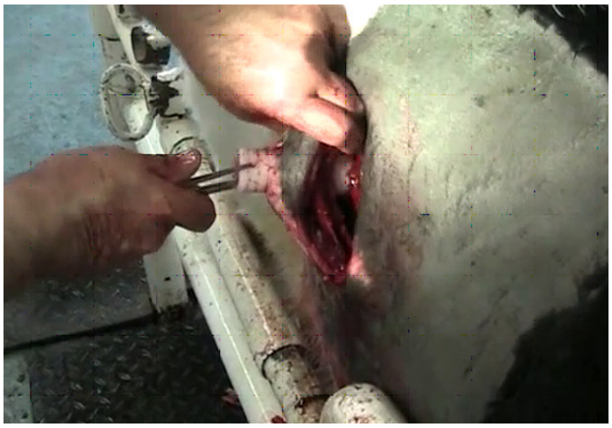


Figure 6. Guiding of the cannula out of the abdominal wall

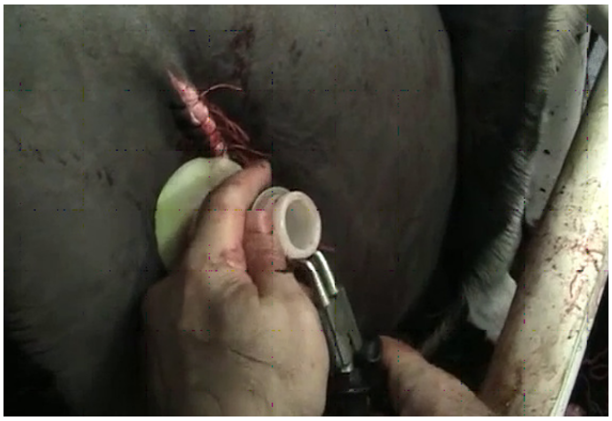


Figure 7. Fixation of the cannula's tube

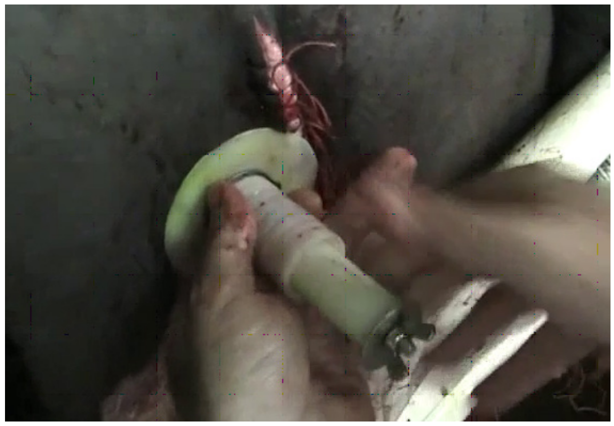


Figure 8. Closing of the completed cannula

Hané) are applied regularly in the form of injections and as preventive treatment of the liver, Menbuton "WERFT" (Sanochemia Pharmaceutika AG) is applied.

Post-operative care

After the surgery, high quality hay is provided for 2 days until the atony of the rumen, caused by xylazine, passes away. Then the gradual acclimation to experimental diet (minimum 14 days) begins. Antibiotic shield (Norostrep inj.) in addition to the day of surgery for 4–5 days more or after two applications of Norostrep, on the third day depot Shotapen inj. is applied. In case of swelling, the area of the surgical wound is treated with resorption ointment (Aphlegmin ung.), avoiding the site of the incision and sutures. The skin sutures are removed on the 8^{th}–10^{th} days after the surgery. On the day the sutures are deleted, the plastic base is removed. Complete cleaning of the area surrounding the tube is performed. In case of necrotic tissue occurrence or emergence of the outer intestinal circular suture, those are carefully removed using tweezers and by washing with warm physiological solution.

Adhesions are sufficiently firm, there is practically no risk in careful manipulation. The cleaned base is placed back on the tube and fixed by metal sleeve. This process, in addition to regular washing of the tube's surroundings, needs to be repeated regularly at least twice a week. It is important to permanently coat the skin under the tube with ointment (Indulona). After the surgical wound is healed, it could get infected. If the animal lies on its side, where the cannula emerges, a small amount of acidic duodenal chyme can escape, which contaminates the fixation bandage and irritates the skin. A good method of protecting the site of the cannula's emergence is the application of chlorophyll (Chrorophyllum spray). Other dermatologics can also be used (Infandolan ung., Panthenol spray, Indulona) in the form of ointment or cream. Permanent protection of the skin at the site of cannula's emergence is especially important.

It is necessary to remember daily physical contact of the personnel with the site of cannula's emergence. This "training" entirely eliminates later defensive movements and the animal's stress. Following this procedure, animals can be used in experiments for a number of years without issues.

RESULTS AND DISCUSSION

The technique of rumen fistulation and intestinal cannulation in large and small ruminants has been known for decades (Dougherty, 1955). This surgical technique, except for minor modifications, is still used today. The development was recorded mainly in the methods of intestinal chyme collection. When all-day flow needed to be measured, re-entrant cannulas are used. However, those required not only intensive care to avoid clogging of the bridge, but also laboriousness during the actual collection of the chyme in the experiments. Attempts to automate the work process have not changed anything (Ivan *et al.*, 1985; Wanderley *et al.*, 1985). Therefore, closed and open T-cannulas were gradually introduced, which, like bridge cannulas, also provided the possibility of flow measurement (division of the tube or balonometry). This greatly simplified the procedure, as the need for intestinal resection and bowel formation was eliminated (Komarek, 1981; Robinson *et al.*, 1985; Robinson and Kennely, 1990).

A transitional type between these was a cannula designed by Ivan and Johnston (1981). This development was dictated not only by new methodological options, but also by the discovery of new inert materials usable for the production of cannulas from total collection of chyme (Aliev, 1974; Rohr *et al.*, 1984; Schwarzenböck *et al.*, 1983; Girschewski *et al.*, 1972), through intermittent sampling with the use of markers (Kaushal and Swan, 1982; Wanderley *et al.*, 1985; MacRae and Ulyatt, 1972), to the use of mobile bags methods. The last one has several advantages over the previous ones. It does not require the animal to be fed the test feed alone throughout the accommodation and experimental period. Therefore, the minimum amounts of samples enclosed in bags are sufficient for testing, and the experimental animal is only a donor of a suitable environment that allows the experimental feeds to be exposed to microbiological and enzymatic processes in the digestive tract. This fact also allows the testing of hazardous by-products and waste products of industry, which could lead to intoxication when fed in the feed ration. Another advantage is that several feeds can be tested at the same time.

The use of a combined *in situ* method (rumen, duodenum) is particularly suitable for testing physically and chemically treated feeds, where the degree of treatment to protect crude protein from microbial degradation in the rumen (bypass protein) may lead to a consequent reduction in their utilization in the gut. During these experiments it was found that each feed showed an individual sensitivity of crude protein depending on the type and intensity of treatment (Szakács, 1989). Thanks to the possibility of determining the appropriate level of feed treatment, the standardized combined *in situ* method has found wide application in nutritional experiments (Chrenková *et al.*, 2012; 2018).

In addition to the rumen fistula (Szakács *et al.*, 1990a, b; Szakács *et al.*, 2021) needed to determine potential degradability, a simple closed T-cannula is sufficient to determine intestinal and total digestibility. After incubation of the test feed in the rumen and incubation in artificial spleen fluid (trypsin, HCl), the residues are placed in sealed bags into the proximal duodenum. These bags, after passage through the intestinal tract together with the chyme, are then captured by washing the faeces through a sieve with suitable openings.

Harmon and Richards (1997) give a very good overview of the development of the types of cannulas and the materials from which they were made. Due to the lack of other options, we currently use patented flexible rumen cannulas and hard intestinal open T-cannulas made in the USA (Bar Diamond, Inc.). In the past, we have successfully used flexible cannulas made of PVC paste, designed by Girschewski *et al.* (1972). We applied them in layers on a suitable matrix and polymerized by heat. Their disadvantage was that after a long period of use, they hardened, and we always had to produce new ones for new animals for the procedure. Their main advantage was that the cannula tube, led through the skin, could be easily fixed with a plastic ring and gauze. After being torn out of the intestine or ruptured, such a cannula could easily be replaced by a replacement cannula, which softened after immersion of the fixation base in hot water. After running out of the paste, despite the description of other options (Buttle *et al.*, 1982), we could not replace them, due to the absence of manufacturers. We, therefore, consider hard plastic T-cannulas, the only ones available on the market, that we now use, to be a step backwards. From the viewpoint of the complexity of the procedure, implantation of a T-cannula into the duodenum is more complicated than rumen fistulation. In terms of physiological consequences, it is exactly the opposite. In the duodenum, while maintaining the permeability and motility of the intestine, complications are minimal. During rumen fistula, on the other hand, disruption to the anaerobic environment may be caused, as well as contraction disorders and the recovery takes weeks.

The cannulation itself follows the method described by Komarek (1981), but the base of the cannula is not fixed in the duodenum using lock-stitch semi-muscular sutures. We consider more reliable doubled oval circular sutures, which after the removal or insertion of the remaining intestinal mucous membrane, when the outer suture is tied off, do not cause problems with serum sweating and offer better fixation of the cannula's tube as well. The second difference is the modification of the cannula itself by filing down of peritoneal ring (Figure 1a, b) and elimination of the "protective sleeve", which is sewn around in strips around the entire circumference of the intestine after segmentation of mesenterium. It is hardened fluroformalin foam. Komarek (1981) cites as example Ivan and Johnston (1981), who used a similar ring from vein polyester prosthetic in sheep. They explained the need for the ring by better fixation of the cannula's base in the intestine. The assumption that the ring, after its gluing and growing to the intestine and peritoneum using fibrin, would prolong the utilization of the animals in experiments, was not confirmed in our case. Cannulated animal's organism identified the vein prosthetic ring as a foreign object and rejected it three months after the surgery. A part of the ring emerged through the skin along with the proximal part of the intestinal cannula. As a result, the animal had to be euthanized. It was an original cannula for sheep designed by Ivan and Johnston (1981) that we tested to create a similar one for large ruminants. The omission of this phase of the operation proved successful, because after leading the tube through the skin a solid ligament stoma formed. This procedure allowed for long-term survival of the cannulated animals. Of the more than two dozen operated large ruminants, in only one case was the base of the cannula ripped from the intestine after mechanical insertion. But even in this case, thanks to a solid ligament stoma, we managed to replace it with a flexible PVC cannula after dissecting the fixed cannula using a skin incision.

The only disadvantage of the described procedure is a slight outflow of intestinal fluid at the outlet of the tube, which irritates and macerates the skin due to the low pH of the duodenal chymus. This problem is solved as described in the guidelines for post-operative care. We consider observance of the relatively broadly described principles of animal care before and after the operation even more important than the operation itself. They guarantee that cannulated animals can be used in experiments for many years. Even today, we have animals operated more than 15 years ago, which are no longer included in the experiments themselves but used as donors of rumen and intestinal fluid for research purposes.

Russel's principle of 3 Rs (The principles of Humane Experimental Technique), mentioned in the Abstract, was later expanded by Tannenbaum and Bennet (2015), who cite the aforementioned literature. Methodology research is performed in accordance to this principle in order to examine the possibilities to replace surgically-altered experimental animals by other, especially laboratory techniques (Stern *et al.*, 1997; Ipharraguerre *et al.*, 2007). Most authors publishing today, reference the early technique and the methodology defining experimental animals (breed, age, sex, weight) or the type and material of the cannula used.

CONCLUSION

Mastering duodenal cannulation technique in cattle, as a second step after rumen cannulation (Szakács *et al*., 2021), allowed a seamless and long-term use of experimental animals in experiments with nutrient degradability and digestibility, *in situ* and method of the mobile bags (Chrenková *et al*., 2012, 2018).

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