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SHORT COMMUNICATION

Successful Development of a Fetal Ovine Model for Esophagus Tissue Engineering

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ABSTRACT

In esophagus tissue engineering, large animal models need to be developed to generate tissues of substantive size that can be applied in the future for clinical applications. The ovine fetal model has been successfully developed in this pilot study for esophagus tissue generation. Fetus (n = 5) was operated between the 120 and 125 days of gestation (term 140 days) with a longitudinal neck incision through which the esophagus was exposed. Full-thickness esophagus biopsies were obtained after which an end-to-end anastomosis was performed. Organoid units isolated from biopsies were seeded on collagen scaffolds to create tubular constructs which were implanted into the fetal omentum. The fetuses were returned and the pregnancy continued. There was no mortality and all five lambs were delivered at term. "Rudimentary" tissue engineered esophagus was generated using the omentum as an *in situ* bioreactor. The ovine fetal model for esophagus tissue engineering could successfully be established in this pilot study.

Key words: Esophagus; Fetus; Organoid units; Ovine; Scaffolds; Tissue engineering

Tissue-engineered esophagus requires investigations to be performed on large animal models before clinical applications. The possibility of generating substantial tissue that could be utilized for esophageal replacement in large animal models is being explored [1-3]. Preliminary investigations from our group developed protocols for the isolation and culture of ovine esophageal epithelial cells [4,5], which were pre-seeded on collagen scaffolds and to create hollow tubular constructs that were implanted in the lamb omentum to provide *in situ* bioreactor conditions for the generation of vascularized rudimentary esophagus [6]. At the same time, investigations are being also performed with regard to identifying the suitable biomaterials for esophageal tissue engineering [7,8]. With the lessons learnt from the lamb model and to offer further surgical advantages, the suitability of ovine fetal model for esophagus tissue engineering through this pilot study was investigated.

Experiments were performed under the guidelines of the Animal Ethics Committee, Ministry of Science and Research, Vienna on fetuses (120–130-day-old) from Austrian Mountain Sheep. After induction of general anesthesia in the ewe, a sagittal lower abdominal laparotomy was performed and the uterus was exposed (Figure 1a). The fetus was located and the head was exteriorized after opening the amniotic cavity (Figure 1b). A nasogastric tube was passed through the fetal esophagus into the stomach. A longitudinal neck incision was performed and the neck muscles were carefully dissected to expose the trachea and the esophagus. Identification of the esophagus was aided by the palpation of the nasogastric tube. After the placement of stay suture, a segment of the esophagus was resected to obtain organoid units (OUs) (see protocol below) (Figure 1c). The esophagus segments 3 cm long were resected in all the five fetuses operated. The two ends of the esophagus were anastomosed end-to-end with vicryl 5-0 sutures over the nasogastric tube (Figure 1d). After completion of the anastomosis, the nasogastric tube was removed and the cervical skin incision closed with absorbable subcutaneous sutures and non-absorbable cutaneous suture.

Fetal esophagus OU's were isolated with slight modifications to a protocol reported for rodent esophageal OU's isolation [9]. Esophagus OUs were produced by dissect-

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ing the fetal cervical esophagus biopsy into full-thickness 2 mm × 2 mm sections after lengthwise opening. The resected specimens were washed two times in 4°C Hanks balanced salt solution, sedimenting between washes, and digested with 0.25 mg/mL dispase (Boehringer Ingelheim) and 800 U/mL collagenase Type I (Worthington) on an orbital shaker at 37°C for 20 min. The digestion was stopped with three 4°C washes of a solution of high-glucose Dulbecco's modified Eagle's medium, 4% heat-inactivated fetal bovine serum, and 4% sorbitol. The OUs were centrifuged between washes at 150g for 5 min, and the supernatant was removed. OUs were reconstituted in high-glucose Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum, counted with a hemocytometer, loaded at 300,000 units per polymer at 4°C, and maintained at that temperature until implantation, which occurred in <1.5–2 h.

The OUs were seeded using the "drop-on" technique on native bovine collagen scaffolds cross-linked with glutaraldehyde (Symatese Biomateriaux, Chaponost, France) delivered in sterile packages. The sponges are highly porous with the pore size in the range of 100 μm. Individual scaffolds were prepared (40 mm × 40 mm in size and 2 mm thickness) before being sterilized with 22.5 kGy β-radiation. The seeded scaffolds were fabricated into tubes by placing them on 4 cm long sections sterile endotracheal tubes with an outer diameter of 8.8 mm (size 6.5; Mallinckrodt Inc., Hazelwood, MO) to create cell-scaffold construct. To keep the dimensions of the construct comparable to normal lamb esophagus, the endotracheal tube size was determined by the placement of endotracheal tubes of various outer diameters in the esophageal biopsies, with size 6.5 determined to be the most suitable. The edges of the collagen scaffold were sutured using interrupted monofilament absorbable suture 5-0 over the endotracheal tube [10].

The fetuses were now flipped to expose the abdomen. A sagittal laparotomy was performed with care taken to stay approximately 1 cm away from the fetal umbilical cord. The omentum was exposed and the construct was wrapped into the omentum, and the omental edges secured using non-resorbable sutures to (a) secure the construct and (b) to allow better identification later at the time of retrieval (Figure 2a). The implanted construct and omentum were returned into the abdominal cavity and the laparotomy incised was closed. The fetus was returned into the amniotic cavity and the cavity was filled with saline before closure. The laparotomy incision of the ewe was closed and the pregnancy was allowed to continue.

The lambs were delivered normally without interventions and were carefully monitored for a period of 3 months (Figure 2b). After this period, euthanasia was induced under general anesthesia for the retrieval of the implanted constructs. The lambs were reoperated and the constructs were removed for histological and morphological evaluations. During the retrieval, the previous laparotomy incision was opened and the constructs localized. Photographic documentation of the area of construct placement was performed before omental resection (Figure 2c). At this time, the constructs were resected from the omentum and the cylindrical edge was exposed to remove the stent. The constructs were then carefully dissected and immersed in phosphate buffer solution before fixation for histological examination which showed the presence of viable esophageal epithelial cell on the construct (Figure 2d).

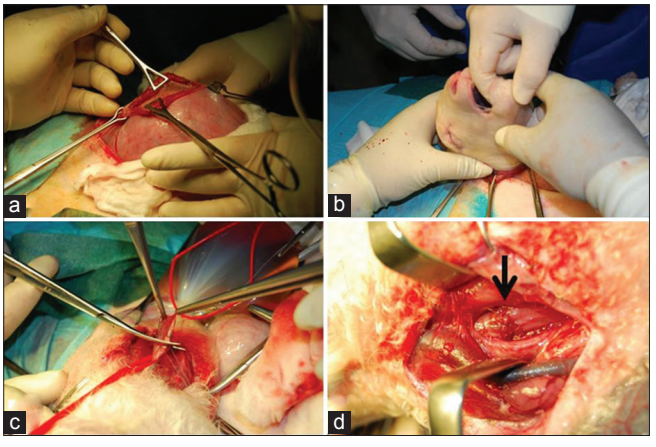

Figure 1: (a) After a lower abdominal sagittal incision in the pregnant ewe, the uterus is exposed, (b) the amniotic cavities are opened and the head of the fetus is exposed, (c) a later cervical sagittal incision is made and the esophagus exposed; with 3 cm resection for obtaining organoid units, (d) the ends of the esophagus are anastomosed (arrow) over a underlying nasogastric tube using absorbable 5/0 vicryl sutures

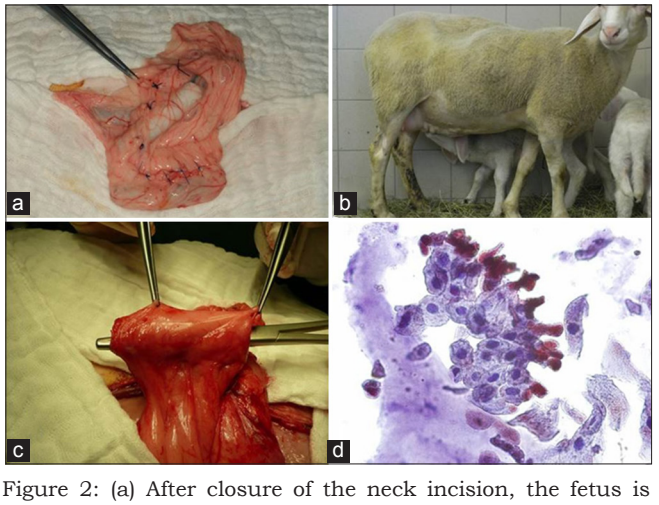

Figure 2: (a) After closure of the neck incision, the fetus is flipped and a middle abdominal sagittal incision is performed to expose the omentum in which stented constructs (with organoid units) are wrapped and securing using non-absorbable sutures. (b) Operated lamb in good general condition drinking milk confirming the patency of the fetal anastomosed esophagus. (c) At 3 months, the retrieved construct can been seen attached to the omentum. (d) Histology of the inner lumen of the construct demonstrating patches of viable ovine esophageal epithelium (Stain: Pan-Cytokeratin 26)

There are many advantages of fetal surgery for esophagus tissue engineering: (1) The esophageal biopsies offer isolation of cells with more proliferative potential from fetus than those obtained from lambs, (2) the esophageal anastomosis performed after removal of the 3 cm esophageal segment in the fetus allows for the segment to heal without the abrasive action of grass feeds that may disrupt the anastomosis in lambs, (3) the fetal model avoids the need for parenteral nutrition after surgery during the time the anastomosis is left to heal, (4) since the pregnancy is allowed to continue for 2 weeks until term birth, the fetal model avoids the placement of a gastrostomy tube which may be necessary in a large animal model, (5) morbidity is low in the fetal ovine model (Postpartum, there was only 1 morbidity, in which a lamb developed a stenosis at the site of anastomosis which was dilated using endoscopic balloon dilators), and (6) the comparatively larger length of the neck in the ovine model offer the possibility of a biopsy through a cervical incision, avoiding the morbidity of a thoracotomy. The successful establishment of the fetal ovine model in esophagus tissue engineering can be further explored in the context of esophageal atresia, a congenital malformation in which large portion of the esophagus is missing in the neonate which at present is reconstructed using various surgical transposition techniques.

Author's contribution

All authors contributed equally in concept, design, literature review, drafting the manuscript, and approval of the final manuscript.

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