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Cryopreserved Human Amniotic Membrane and A Bioinspired Underwater Adhesive To Seal And Promote Healing Of Iatrogenic Fetal Membrane Defect Sites

Ramesha Papanna, M.D., M.P.H.¹, Lovepreet K Mann, M.B.B.S.¹, Scheffer C.G. Tseng, M.D., Ph.D.², Russell J Stewart, Ph.D.³, Sarbjit S Kaur, Ph.D.³, M Michael Swindle, D.V.M.⁴, Themis R Kyriakides, Ph.D.⁵, Nina Tatevian, M.D., Ph.D.⁶, and Kenneth J Moise Jr, M.D.¹

¹The Fetal Center, Department of Obstetrics, Gynecology and Reproductive Sciences, The University of Texas Medical School, Houston, TX

²Research & Development of Tissue Tech, Ocular Surface Center, P.A., Miami, FL

³Department of Engineering, College of Engineering, The University of Utah, Salt Lake City, UT

⁴Comparative Medicine, Medical University of South Carolina, Charleston, SC

⁵Department of Pathology, Yale University, New Haven, CT

⁶Department of Pathology, The University of Texas Medical School, Houston, TX

Abstract

Introduction—We investigated the ability of cryopreserved human amniotic membrane (hAM) scaffold sealed with an underwater adhesive, bio-inspired by marine sandcastle worms to promote healing of iatrogenic fetal membrane defects in a pregnant swine model.

Methods—Twelve Yucatan miniature pigs underwent laparotomy under general anesthesia at 70 days gestation (term = 114 days). The gestational sacs were assigned to uninstrumented (n=24) and instrumented with 12 Fr trocar, which was further randomized into four different arms-no hAM patch, (n=22), hAM patch secured with suture (n=16), hAM patch with no suture (n=14), and hAM patch secured with adhesive (n=9). The animals were euthanized 20 days after the procedure. Gross and histological examination of the entry site was performed for fetal membrane healing.

Results—There were no differences in fetal survival, amniotic fluid levels, or dye-leakage from the amniotic cavity between the groups. The fetal membranes spontaneously healed in

For correspondence, please contact: Ramesha Papanna, MD, MPH, Section of Maternal-Fetal Medicine, Department of Obstetrics, Gynecology & Reproductive Sciences, The University of Texas Medical School, 6431 Fannin Street, Suite 3.274, Houston, TX 77030, ramesha.papanna@uth.tmc.edu, T: 713 486 6552 / F: 713-500-7860.

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instrumented sacs without hAM patches. In sacs with hAM patches secured with sutures, the patch was incorporated into the swine fetal membranes. In sacs with hAM patches without sutures, 100% of the patches were displaced from the defect site, whereas in sacs with hAM patches secured with adhesive 55% of the patches remained in place and showed complete healing ($p=0.04$).

Discussion—In contrast to humans, swine fetal membranes heal spontaneously after an iatrogenic injury and thus not an adequate model. hAM patches became incorporated into the defect site by cellular ingrowth from the fetal membranes. The bioinspired adhesive adhered the hAM patches within the defect site.

Keywords

Fetal membrane healing; preterm premature rupture of membranes; fetal surgery; regeneration; underwater adhesive

Background

Despite the recent advances in invasive fetal surgeries have improved fetal outcomes, iatrogenic preterm premature rupture of membranes (iPPROM) remains a major complication leading to premature delivery undermining the complete benefits of such surgeries^{1,2}. iPPROM has been attributed to the non-healing nature of human fetal membranes^{3,4} and chorioamnion separation at the site of entry⁵. Numerous *in vitro* and *in vivo* efforts to repair iatrogenic fetal membrane defects have been reported using various sealants and plug materials, including fibrin based products⁶, collagen slurries, blood cryoprecipitates, platelets⁷, collagen sponges, and decellularized tissue scaffolds^{8,9}. *In vitro* experiments suffered from poor experimental design with respect to replicating the wet amniotic environment. Previous *in vivo* studies conducted in rabbits were limited in their ability to study complete wound healing due to the short 28 day gestation period. In our retrospective studies, plugging the fetoscopic entry port with gelatin sponge material after laser surgery did not decrease the incidence of iPPROM compared to laser surgeries in which sealants were not used.^{10,11} There is no proven method available for humans that reduces the incidence of iPPROM.

Cryopreserved human amniotic membrane scaffolds (hAM) have been used in ophthalmology as a permanent graft to fill in tissue defects that allowed integration of host cells into the defect, and as a temporary biological bandage to facilitate wound healing by suppressing excessive surgical or disease-induced host tissue inflammation¹². Due to hAM's inherent anti-inflammatory and anti-scarring properties, it has been used in orthopedic applications to decrease local inflammation and adhesion formation following tendon^{13,14} and nerve repair¹⁵.

The water-borne adhesive used in this study was inspired by the undersea glue of sandcastle worms¹⁶. To create a synthetic biomimetic adhesive, the chemistry of the natural glue was mimicked with sets of oppositely charged polyelectrolytes synthesized with the same side chain chemistry (phosphates and primary amines) in the same molar ratios as the natural glue proteins¹⁷. The bioinspired adhesive has several ideal properties as an injectable wet-

field adhesive. Most importantly, the oppositely charged PEs associate electrostatically and condense into a concentrated fluid macrophase in a narrow range of solution conditions. Although the individual polyelectrolytes components are highly water soluble, the condensed polyelectrolyte macrophase is slowly miscible with water, and therefore does not dissolve or disperse into physiological fluids, including blood¹⁸ and amniotic fluid, on a time scale of hours. As a result, the water-borne adhesive remains at the application site during the curing process even when fully submerged in water. In a previously published study, the feasibility of using the synthetic adhesive with hAM scaffolds to seal wet and submerged fetal membrane defects was demonstrated *in vitro*¹⁹. In that study, the synthetic adhesive was shown to be non-cytotoxic using live *ex vivo* human amniotic membranes. A similar condensed polyelectrolyte adhesive formulation was biocompatible and effectively secured and maintained alignment of rat skull fragments during healing²⁰.

In this study, we used a swine model, which has gestational age of 114 days, to observe changes at the trocar site between 18 to 21 days post-surgery to understand the process of wound healing. Two hypotheses were tested in two different phases of study, Phase I: hAM patches promote fetal membrane healing after an iatrogenic defect compared to no hAM patch, and Phase II: underwater adhesive stabilized the hAM patch at the site of defect to promote healing.

Materials and Methods

Human amniotic membrane scaffold and underwater adhesive

Research grade hAM was kindly provided by Bio-Tissue, Inc. (Miami, FL)¹⁹. The adhesive was prepared as previously described²¹. The details of the methods of preparation are in the supplementary material.

Animal study

The study protocol was approved by the Institutional Animal Care and Use Committee (AWC-12-038) at The University of Texas Health Science Center at Houston. All animal care was in compliance with the Guide for the Care and Use of Laboratory Animals. The animal facility is accredited by Association for Assessment and Accreditation of Laboratory Animal Care - International, and the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The facility meets all standards mandated by the Animal Welfare Act, Centers for Disease Control, National Research Council Guide for the Care and Use of Laboratory Animals.

Pregnant Yucatan miniature swine were obtained from Sinclair Bio-Resources (Columbia, MO). Time-mated, pregnant Yucatan swine arrived at the facility one week prior to surgery for acclimatization. The animals were cared for by trained and experienced veterinary technicians supervised by board-certified veterinarians. The animals had free access to food and water except for the 12-hour period directly preceding surgery. The surgery was performed at 70 days gestation (term: 114 days) under general anesthesia using isoflurane inhalation. The list of medications for pre-, intra- and post-operative periods are listed in Table 1. Indomethacin was administered as a rectal suppository before surgery and intra-

operative terbutaline was given by intravenous pump for tocolysis. Medroxyprogesterone acetate intramuscular injections were given as a supplement for 4 days to prevent post-surgical luteal deficiency. At the time of the surgery, the pregnant sow was placed in the left lateral position to prevent compression of the inferior vena cava. Local anesthesia was administered with a subcutaneous injection of 20 cc of 0.25% bupivacaine on the right side lateral to the mammary glands followed by vertical laparotomy incision. The uterine horns were exposed with gentle manipulation. The gestational sacs that were closest to the cervix were not instrumented (negative controls). The remaining sacs were instrumented. The surgeries were performed in 2 study phases. In phase I, the gestational sacs from 6 animals were randomized into two groups: instrumented without a hAM scaffold placement, and instrumented with a hAM scaffold secured in the defect with sutures. In phase II, the sacs were randomized into two groups: instrumented with hAM scaffold placement without sutures, and instrumented with hAM scaffold placement secured in the defect with adhesive.

The instrumentation to enter the amniotic cavity for all sacs was performed in the similar manner as in humans for percutaneous approach during a fetoscopy^{10,22}. Under ultrasound guidance the entry site was chosen on the antimesenteric side of the uterine horn to avoid the allantois. An 18 gauge echo tip needle (Cook; Bloomington, Indiana, USA) was inserted into the gestational sac under ultrasound guidance. This was followed by J-wire insertion, after which a 12 Fr trocar (Cook; Bloomington, Indiana, USA) and cannula were threaded over the J-wire. The entry into the gestational sac was confirmed by the back flow of amniotic fluid through the cannula. Ten cc of amniotic fluid from each instrumented sac was collected. For the instrumented with hAM placement sacs, a 4 by 4 cm square patch of hAM scaffold was folded into an “umbrella” shape with the chorionic surface facing out.¹⁹ The optimal patch size to occlude the 12 Fr cannula and the method of delivery has been described in our previous publication.¹⁹ A 4-0 monocril suture was tied to the center of the fold. The hAM patch was loaded into the 10 Fr cannula with a 8 Fr blunt trocar to push the patch. The unit was introduced into the 12 Fr operating cannula and the patch was displaced into the amniotic sac. Both cannulas and the trocar were withdrawn leaving behind the hAM patch in the amniotic sac with the retrieval suture coming through the uterine entry site. Gentle traction was applied to the suture to draw the hAM patch into the trocar defect site.

In instrumented sacs sealed with hAM and sutures, a 4-0 monocril suture was placed through the uterine wall into the patch to secure it to the uterine wall. In instrumented sacs sealed with hAM without sutures, the excess retrieval suture was cut close to the patch and no fixation was used. In instrumented sacs sealed with hAM and adhesive, part of the patch was pulled into the incision and the activated adhesive was applied between the patch and the uterine wall. The retrieval suture was cut close to the patch.

All sacs were marked with different colored segments of pediatric feeding tubes sutured to the uterine serosa <1 cm away from the site of entry for identification at the time of harvest. On postoperative day 20 (term: 114 days), the animals were sedated before laparotomy. The latency period of 21 days after the initial surgery provided sufficient time to study complete wound healing of fetal membranes. Fetal viability was confirmed using an ultrasound. Then, the animals were euthanized using intravenous Beuthanasia-D (phenobarbital sodium and phenytoin sodium). The amount of amniotic fluid was quantified using ultrasound guidance

by measuring the maximum vertical pocket. Presence of amniotic fluid volume with maximum vertical pocket of >1 cm was considered as normal. The amniotic fluid was withdrawn for future studies. A dye test was performed with injecting methylene blue under the ultrasound guidance into the amniotic cavity to examine for the leakage at the site of defect between the fetal membrane layers and the uterine wall. Hysterotomies were performed on the mesenteric portion of the uterine horn to enter the sac. The uterine entry site was identified by the tag that was placed during the initial surgery. The corresponding fetal membranes were visually examined for separation of chorion from the underlying endometrial lining, continuity at the site of defect, signs of healing and presence of hAM. An en-bloc section of 4-5 cm in diameter of the uterine wall and membranes from the negative controls and all arms of treatment site were taken.. The specimens were fixed in 10% buffered formalin for 24 hours. Sections were performed every 3 mm and were embedded in paraffin. The blocks were sliced at 4 μ m thickness for histological examination using H&E and trichrome staining.

The sample size was calculated prior to conducting the experiment. The sample size for the investigation was calculated with a prediction of lack of membrane integrity in 80% of punctured sacs and an 80% chance of integrity after a therapeutic endeavor to seal the defect, with an 80% power to detect the difference and a p-value of 0.05. The 80% lack of healing was based on data from other animal studies^{9,23,24} and our preliminary study in swine of 2 animals delivered at 5 days after the procedure. This calculation required a total of 48-instrumented sacs with 12 sacs in each of the 4 arms of the study. The average size litter in the Yucatan breed was reported to be 6 fetuses. The lower 2 fetuses, located in each uterine horn in each sow, will serve as negative controls (no puncture). Thus, 12 pregnant sows were needed for the study. Anticipating a 50% rate of spontaneous pregnancy loss after the initial surgery and possible less number of fetuses, the total sample size was increased to 30 sows.

Outcome analysis was performed on an intent-to-treat basis. Pearson χ^2 or Fischer's exact was performed for statistical significance, with $p < 0.05$ considered significant.

Results

The results from both phase I and II are illustrated in Table 2. In each phase, surgeries were performed on 6 sows. In phase I of the study, there were 2 out of 6 sows (33%) that demonstrated signs of preterm labor 5 and 6 days after the surgery. These sows were euthanized and tissues were immediately harvested. During necropsy, one sow demonstrated an inadvertent entry into the sac most proximal to the cervix at the time of trocar insertion. In the second sow, the allantois was noted to have been disrupted in 2 sacs when the trocar was inserted. Aerobic and anaerobic cultures from the most distal sacs grew *Staphylococcus chromogens*, a normal vaginal flora of swine, which suggested an ascending infection had occurred. In both animals, the sites of entries were examined and were able to analyze the healing of fetal membranes successfully. Thus, the findings were included in the analysis. The other 4 sows were euthanized three weeks after the procedure as planned. There were no significant differences in the fetal survival, amniotic fluid levels and leakage of dye between the two treatment groups.

In the phase II of the study, the last 4 sows had demise of all fetuses except for two fetuses. These fetuses were in the last two animals, one in each animal, and both sacs had been treated with hAM patches and adhesive. The examination of the demised fetuses showed maceration of various stages, suggesting the most likely time of death was 1-4 days after the procedure. In the sacs sealed with a hAM patch without sutures, none of the hAM patches remained in place in the uterine wall. In sacs sealed with a hAM patch and adhesive, 5 out of 9 (55%) of the patches were present in the defect site in the uterine wall 3 weeks after the surgery ($p < 0.004$). In the sacs with live fetuses, the defects were completely healed. In hAM-adhesive there was cellular ingrowth into the patch. The membrane integrity and dye leakage, the testing could not be performed due to the maceration of membranes in these sacs.

Gross examination of the uterine wall is illustrated in Fig. 1A and 1B, the serosal surface and amniotic surface, respectively, of an uninstrumented sac are shown. The amniotic surface had multiple rugae and chorionic nodules (*black arrow*). Fetal vessels in the fetal membrane were easily visible (*green arrow*). In Fig. 1C and 1D, the serosal and amniotic surfaces, respectively, of the instrumented site of an instrumented sac patched with hAM and sutures are shown. The tag placement site and the trocar entry site are indicated by the black and yellow arrows, respectively. The visible entry site in the amniotic membrane had scar tissue and was healed completely (*red arrow*). In Fig. 1E and 1F, the serosal and amniotic surfaces of trocar entry site of a sac sealed with a hAM patch and sutures are shown. The hAM patch was evident within the fetal membrane defect with evidence of continuation of amniotic membrane over the patch (*red arrow*).

The histological differences between the groups in Phase I are illustrated Figure 2. In Fig. 2A, the uninstrumented control fetal membrane had the normal relationship between the uterus and fetal membranes. Fig. 2B is a higher magnification image of the red boxed area in Fig. 2A. The amniotic membrane (Am) appeared thin with a single layer of epithelium (*red arrow*), whereas the chorion (Ch) was attached to the epithelial layer of the endometrial and the placentation was “epitheliochorial” type. This appeared like a “tram-track” on histology (*black arrow*). There were multiple small and larger fetal vessels in the fetal membranes (V). The chorionic layer appeared spongy and the chorionic epithelium abutting the epithelium of the endometrium appeared in a “tram-track” fashion. Fig. 2C and 2D are higher magnification images of the cut section of the uterine wall through an instrumented sac without a hAM patch. The amniotic membrane exhibited loss of rugae (*red arrow*), which was present in abundance in the uninstrumented control. The fetal vessels in the amnion were thinner. There was complete continuity of the fetal membranes at the site of defect. In Fig. 2D, the boxed region of Fig. 2C at higher magnification, the chorion was detached from the endometrium at the healing site (*black arrow*). The uterine wall had evidence of a healed wound with collagen deposition (*yellow arrow*). The wound surface of the amnion appeared healed with scarring. In Fig. 2E and 2F at higher magnification, the histological section of uterine wall with a sutured hAM patch was seen. The hAM patch (*orange arrow*) was seen as thin folds of membrane integrated into the fetal membrane defect. The amniotic epithelium, chorion and endometrium were continuous over the

scaffold (*red arrow*). The homogenous blue staining suggests incorporation of hAM into the local host tissue.

In phase II, the histological findings of instrumented sacs plugged with a hAM patch without sutures were similar to the instrumented sacs without hAM patches of phase I. In other words, the instrumented fetal membranes healed spontaneously without a hAM patch. In sacs plugged with hAM patches and adhesive (Fig.3 and inset) the presence of the hAM and its complete incorporation into the local tissue (*red arrow*) and neovascularization (*black arrow*) can be seen. The inset illustrates a higher magnification (60×) image of the red boxed area. The adhesive was not apparent around the scaffold, which suggested the adhesive was absorbed. The absence of histological evidence of the adhesive was not expected since the polymethacrylate-based adhesive is biodegradable. There were no inflammatory cells noted in the hAM, both with suture and with adhesive, suggesting no foreign body or xenographic reaction.

Discussion

Principal findings of the study

In contrast to humans^{3,4}, swine fetal membranes healed spontaneously after an iatrogenic injury. The hAM patches were incorporated into the fetal membrane defects by ingrowth of amniotic epithelium and continuation of the chorion and endometrial layers onto to the scaffold. Sealing hAM patches into the fetal membranes defects with a bioinspired underwater adhesive was much more effective at maintaining the hAM patch in the defect site than not using adhesive. The apparent increase in fetal loss in the animals treated with the adhesive requires further study.

Swine membranes heal injury after surgery

Fetal membranes in swine showed complete healing with minimal scar formation after a trocar injury. This was an unexpected finding. This is possibly due to epitheliochorial placentation in swine compared to hemochorial placentation. Additionally, the swine amniotic membrane has vasculature compared to humans²⁵. Studies in rabbits have shown fetal membrane integrity was maintained in 25-45% of instrumented sacs without plug or scaffold placement^{9,23,24}. However, these studies did not demonstrate complete spontaneous healing at the defect site. This may be due to the limited period of observation in the short pregnancies with a latency of only 3-7 days. In the swine model, the pregnancy was followed up to 3 weeks after the creation of the defect to observe natural healing. Defect size of 14 Gauge heals by secondary intention, which takes place up to 2-3 weeks from injury^{26,27}.

hAM incorporated into the fetal membranes of swine

We observed that a hAM patch was incorporated into the damaged fetal membranes of swine with ingrowth of different layers. These finding are an extension of previous studies which showed proliferation of cells into human amniotic membrane scaffold in fetal membrane defect sites in rabbits^{24,28}. Human amniotic membrane has been used as a regenerative graft in burn injury, corneal injury, cortilagenous injury and dural repair¹²⁻¹⁵.

These were the first large animal experiments designed to study fetal membrane healing for over 3 weeks. Previous studies have examined only up to 3-7 days in rabbit or sheep models. In the phase I study, the fetal survival rates were greater than 80%, which is higher than previously reported animal studies for fetal membrane research of 40-50%^{23,24,28-30}. This higher rate of fetal survival in swine reduces the error in interpretation of fetal membrane healing.

Bioinspired underwater adhesive prevents displacement of the patch from the trocar defect site

The bioinspired adhesive provided sufficient adhesion at the site of defect to keep the hAM patch in place allowing wound healing to take place. Higher fetal demise in phase II versus phase I is not understood. The biocompatibility and cytotoxicity of the adhesive were tested previously in non-pregnant rats to study the fixation and healing of cranial fractures,²⁰ and in *in-vitro* experiments with fetal membrane explants¹⁹.

The previous experiments showed no evidence of adverse systemic effects or localized toxicity. Likewise, the mothers in this study treated with adhesive displayed no adverse systemic effects. In the animals with only one surviving fetus, it was the fetus in the sac sealed with adhesive that survived. In other words, the fetuses in closest proximity to the adhesive survived while all other fetuses were demised, including uninstrumented controls. The gravid uterus is a unique environment and requires context specific safety testing of the adhesive in future experiments. Use of adhesives to secure a patch system in the trocar site is essential as it is impossible to reach the uterine wall through the cannula without a laparotomy.

Swine fetal membranes heal spontaneously. Swine are therefore not an ideal model to study methods to seal human fetal membranes after invasive surgery. Additionally, animal models such as mice, swine and rabbit with multiple gestational sacs do not have preterm premature rupture of membranes or preterm labor as in humans of instrumentation of gestational sacs. Primate fetal membranes do not heal spontaneously after injury and may therefore be a better model³¹. hAM is an effective patch material that may promote fetal membrane healing when secured in the defect site. With continued development, guided by the reported findings, the bioinspired underwater adhesive and hAM patch may become an effective method to treat iatrogenic fetal membrane defects. In the absence of fetal membrane healing, sealing fetal membranes with a patch and adhesive may still significantly reduce the incidence and complications of iPPROM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Fetal membranes in swine model heal spontaneously.
- Human amniotic membrane scaffold integrates into the fetal membrane defect.
- Bio-inspired adhesive adheres the human amniotic membrane scaffold to the fetal membrane defect successfully.

Figure 1. Gross examination of uterine serosa and amniotic surface

A- Serosal surface of uninstrumented; B- Amniotic surface of uninstrumented sacs, *green arrow*- Fetal blood vessel in amniotic membrane, *black arrow*- nodules in fetal membrane; C- Serosal surface of instrumented without hAM, *black arrow*- tag on the serosal surface, *yellow arrow*- healed serosal surface. D- Amniotic surface of instrumented without hAM; *red arrow*- healed fetal membrane defect; E- Serosal surface of hAM with suture; F- Amniotic surface of hAM with suture, *red arrow*- scaffold in fetal membrane defect.

Figure 2. Histological examination of en-bloc sections of uterine wall using Masson's Trichrome staining

Am- amnion, *Ch-* chorion, *En-* endometrium, *myo-* myometrium (uterine wall), *v-* vessels. A and B (high magnification) - uninstrumented sacs, *red arrow-* thin amniotic epithelium, *black arrow-* epitheliochorial placentation; C and D (60X magnification)- instrumented without hAM, *red arrow-* thin endometrium; *black arrow-* chorion decidual separation, *yellow arrow-* scar tissue in the uterine wall; E and F (High magnification)- hAM with suture, *orange arrow-* hAM, *red arrow-* amniotic epithelium growing over the hAM.

Scale: 50 μ m

Figure 3. Masson's Trichrome histology of uterine wall of hAM with UAC
Am- amnion, *Ch-* chorion, *En-* endometrium, *myo-* myometrium (uterine wall). Inset-high resolution image, *red arrow-* hAM; *black arrow-* new blood vessels inside the graft.

Table 1
List of medications for the surgery

One day before surgery:

- Medroxyprogesterone 50mg IM
- Indomethacin 50mg rectal suppository

Day of surgery: All drugs given are calculated on non-pregnant weight (minus 10kg)

Pre-operatively

- EMLA cream (lidocaine 2.5% and prilocaine 2.5%) applied to injection sites 30 to 45 minutes prior to sedation to minimize discomfort
- Telazol (tiletamine/zolazepam 100mg/ml) 4-8mg/kg IM
- Famotidine 0.25mg/kg SC
- Naxcel (ceftiofur sodium) 3mg/kg IV
- Lactated Ringers 10ml/kg/hr IV
- Duramorph (morphine) 1mg in 10 ml of normal saline epidural

Intra-operatively

- Terbutaline 2.5mcg/min IV infusion
- Bupivacaine 0.25% 20 ml for subcutaneous injection
- Isoflurane 2-3% inhalation

Post-operatively

- Naxcel (ceftiofur sodium) 3mg/kg IV
- Excede (Ceftiofur Crystalline Free Acid 100mg/ml) 5mg/kg IM
- Buprenorphine 0.01mg/kg subcutaneous injection

Post-op Day 1 – 4:

- Buprenorphine 0.01mg/kg subcutaneous injection q8 hours x 1 day, PRN thereafter
 - Medroxyprogesterone 50mg IM on Day 2 and Day 4
-

Table 2
Comparison of different interventions in the gestational sacs at 21 days after the primary surgery

	Phase I (Swine #1 - #6)			p-value	Phase II (Swine #7 - #12)			p-value
	Uninstrumented	Instrumented (no hAM)	hAM-suture		Uninstrumented	hAM-no-suture	hAM-underwater adhesive	
Latency period (days)		20 (5-21)				18 (16-21)		
Number of gestational sacs per animal		8 (6-10)				6 (4-8)		
Miscarriage (animal)		2/6 (33%)*				4/6 (67%)**		0.57
N	12	22	16	-	12	14	9	-
Live fetus	10/12 (83%)	15/22 (68%)	13/16 (81%)	0.51	4/12 (33%)	3/14 (21%)	4/9 (44%)	0.67
Presence of patch	-	-	13 (88%)	-	-	0/14 (0%)	5/9 (55%)	0.004
Maximum vertical pocket (mm) Mean $\pm$ SD	2.2 $\pm$ 1.2	1.9 $\pm$ 1.1	2.2 $\pm$ 1	0.63	2.4 $\pm$ 1.1	3.5 $\pm$ 0.7	3 $\pm$ 1.2	0.61
Membrane integrity	-	15/15 (100%)	13/13 (100%)	0.9	-	3/3 (100%)	4/4 (100%)	0.9
Dye leakage	0/12 (0%)	0/15 (0%)	0/13 (0%)	0.99	0	0/3 (0%)	0/4 (0%)	-

hAM- cryopreserved human amniotic membrane, Numbers are presented as n(%) and median and range.

* Spontaneous miscarriage process had begun with vaginal spotting and the animals were euthanized immediately- quantifiable data was obtained for fetal viability and membrane integrity.

** Swine #9 and #10 had fetal demise of all fetuses without vaginal bleeding or fever and swine #11 and #12 had fetal demise of all fetuses except for 1 fetus per animal, the most proximal sac in the right horns.