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Electrospun silk biomaterial implant for teat obstruction in dairy cattle

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Obstructive teat injuries are common in dairy cows, which consequently lead to reduced milk production and poor milk quality, increased cost of treatment, early culling and thereby, a negative economic impact. The present study was undertaken for the production and standardization of a low cost bio-compatible teat implant made of silk fibroin, known for its versatile utility as a biomaterial. Bombyx mori cocoons were collected and processed to make the silk fibroin solution and were made into mat form of 0.2 mm thickness and a pore size of 2-5 µm by electrospinning technique. A comparative clinical trial was conducted with regular silicone plugs and silk fibroin rolled teat plugs in cases of teat obstruction in dairy cattle. The parameters studied included healing period in days for return to milking, milkability and somatic cell count measured at 0, 10, 20 and 30 days.

Key words: *Dairy Cattle, Implant for Teat Obstruction, Silk Biomaterial*

Milk flow disorders are the predominant problem related to udder health. Alacam *et al*. (1990) reported teat lumen as the common site of obstruction in cattle. Un-hygienic maintenance and improper method of milking are contributing factors for teat obstruction. According to Aruljothi *et al*. (2007), corrective surgery for teat obstruction followed by the use of prosthetic PVC tubes help maintain the patency of teat lumen in cattle. Recent experiments indicate that teat dilators and teat canulae themselves cause injuries and cisternitis in the teat (Querengässer *et al*. 1999).

Bombyx mori silk fibres are composed of two filaments of the protein fibroin, coated by a glue-like protein, sericin. The sericin was identified as a possible source of non-physiological inflammatory reaction, and therefore usually removed by a degumming process. Catto *et al.* (2015) reported that silk fibroin after degumming presented high biocompatibility, excellent mechanical properties, and versatile processability.

The present study was undertaken with the objective to develop and standardize electrospun silk fibroin teat implant and to assess its efficacy in clinical cases.

Materials and Methods

Production of silk fibroin solution

The protocol of Rockwood *et al.* (2011) was adapted. Clean and unsoiled *Bombyx mori* cocoons were obtained from TANSILK Vaniyambadi and were dried after removing the worms (Fig. 1a). 5g of silk cocoon mixed with 0.02 M sodium carbonate solution was boiled for 45 min in a warm water magnetic stirrer. After discarding the water, digested fibers were collected and dried in warm air overnight. These fibers were again digested with 9.3 M Lithium bromide four times the volume of weighed silk, in a hot air oven at 60^0 C for 3-6 hr or until silk was dissolved completely.

The silk fibroin solution thus obtained was purified using Slide Lyzer Cassette 3500 MWCO in distilled water for a total period of 72 hr (Fig. 1b). The resultant solution was carefully taken out of the cassette and centrifuged twice for 20 min at 5-10^0 C, 8800 rpm. The supernatant was collected and 500 µL of it was placed in a pre-weighed plain petridish and kept in hot air oven at 60^0 C for one hour to get the dry weight. Percentage of silk fibroin was arrived using the formula and the solution was refrigerated and stored at 4^0 C for further processing.

Percentage concentration of silk fibroin= dry weight – petridish weight / 0.5

Electro-spinning of silk fibroin

The protocol of Rockwood *et al.* (2011) was adapted. The silk fibroin thus obtained was mixed with polymer polyethylene oxide in 1:1, 2:1, 3:1 and 4:1 ratios for electrospinning for obtaining electrospun fibers. The ratio of 2:1 yielded good

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amount of fibers by electrospinning at the collecting drum as it provided optimal viscosity.

For electrospinning, syringe infusion pump was kept at a flow rate of 0.5 mL/hour and 20 kV DC high voltage current was applied to the needle tip, whereas the distance between the needle tip and collecting drum was kept at 12 cm (Fig. 1c). The electrospun fibers were collected in a thick aluminium foil coated with Teflon. The electrospun fibers were imaged using Scanning Electron Microscope facility at IIT Chennai (Fig. 1d). The fiber mats thus produced were carefully peeled off from the rotatory drum collector and immersed in 90% methanol v/v for 20 min followed by their immersion in ultrapure water overnight to remove PEO (Fig. 1e). The mat obtained was 0.2 mm thick and a pore size of 2-5 μm.

The mats were then dried in fume hood and rolled over the silicone teat plug for clinical trials (Fig. 1f).

Fig. 1a: *Bombyx mori* silk cocoons.

Fig. 1b: Dialysis of silk fibroin with 3500 MWCO Slide Lyzer Cassette with float.

Fig. 1c: Electrospinning of silk fibroin at IIT Chennai.

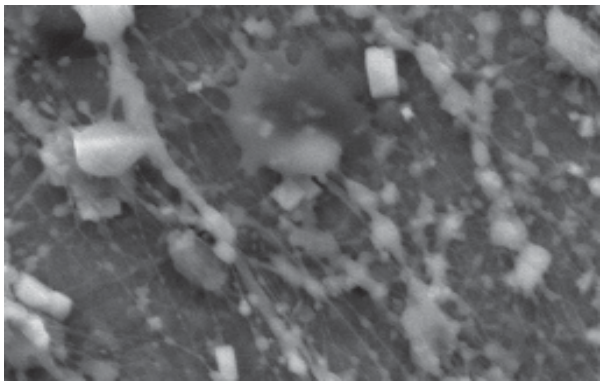

Fig. 1d: Electron microscopic view of silk fibroin.

Fig. 1e: Electrospun silk fibroin sheet with 0.5 mm thickness and 2-5 μm porosity.

Fig. 1f: Electrospun silk fibroin sheet rolled teat plug for clinical trial.

Collection of teat epithelial cells and culture

Teat samples were obtained from slaughter house in a clean manner and were collected and washed in DMEM + antibiotic (Penicillin 25 U/mL and streptomycin 250 µg/mL + Gentamicin 250 µg/mL). Under aseptic conditions, the tissue was scraped with No. 10 BP blade and transferred to 10 mL falcon tube containing the same DMEM + antibiotics solution, centrifuged at 800-1000 rpm for 2 to 3 times and the pellet obtained was suspended in 5 mL of DMEM-FBS + antibiotic medium. Trypan blue staining was done before seeding to see the presence of live cells. The cells were then seeded in the DMEM-FBS with antibiotics.

Trypan blue assay for assessing biocompatibility of the material by direct contact incubation

The cells were seeded in a 6 well plate with electrospun silk fibroin mat pre-dipped in DMEM with antibiotic under laminar airflow. The cells were incubated for 24 hr and the test sample was removed from the 6 well plate using a mosquito artery forceps. Care was taken to avoid scratching of cells. 0.5 mL of trypan blue was added to each well and mixed and incubated for 10 min. The viability of the cells was counted by the presence of cells without the dye and inference was made (Fig. 1g).

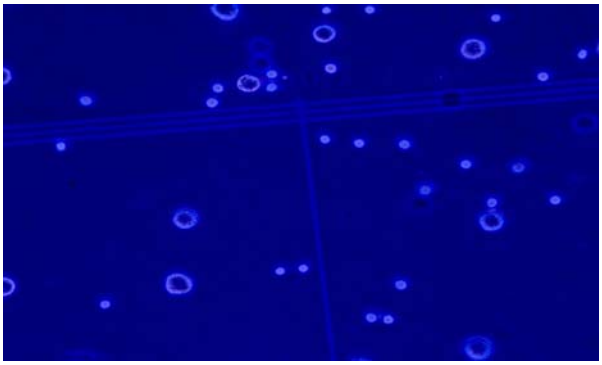

Fig. 1g: Isolation of teat epithelial cells from slaughter house teat samples and its viability by Trypan Blue staining.

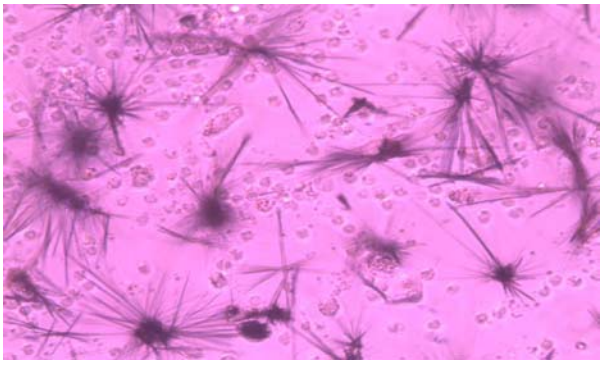

Fig. 1h: MTT assay for biocompatibility.

MTT Assay

100 µL of cells suspended in DMEM medium were added to each well of 96 well plate with electrospun silk fibroin sheet soaked in 10 µL of DMEM. The 96 well plate was placed in CO_2 incubator. On 3rd, 5th and 7th day of incubation, 100 µL of the 12 mM MTT stock solution was added to each well and incubated at 37^{0}C for 10 min. Each sample was mixed again and absorbance was read at 540 nm (Fig. 1h).

Clinical trial of electrospun silk biomaterial implant for teat obstruction

Twelve clinical cases of teat obstruction at the level of streak canal were included in the study under two groups, group I and group II. All the animals were first assessed for the site of obstruction by clinical examination, radiography and ultrasonography (Franz *et al*., 2009). The teat area was surgically prepared with chlorhexidine scrub and Hugh's teat tumour extractor was used to relieve the obstruction under ring block anaesthesia using 2 % lignocaine. To maintain the patency, regular silicone implants were used in group I (Fig. 1i) and electrospun silk fibroin implants in group II (Fig. 1j). Implants were retained *in-situ* with bandage, and parenteral and intra-mammary

Fig. 1i: Application of silicone teat insert.

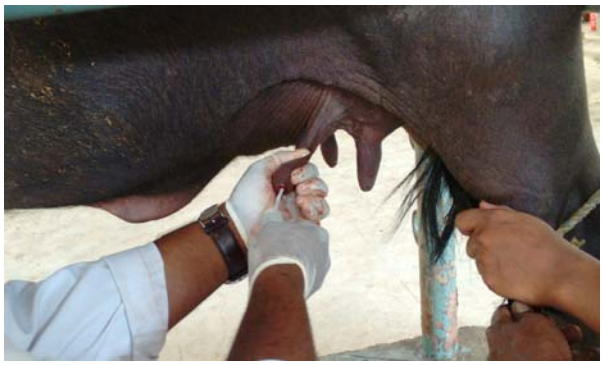

Fig. 1j: Application of electrospun silk fibroin teat implant.

antibiotic infusions were administered. The parameters studied for the comparison of implant efficacy were healing period in days for return to milking, milkability (volume in mL of milk obtained) and somatic cell count measured at 0, 10, 20 and 30 days. The data collected were analyzed statistically using t test. Ultrasonography of the teat and radiographic examinations were carried out to see the healing pattern.

Results and Discussion

Kundu *et al*. (2013) reported that silk based designs allowed easy control on matrix morphology, degradation rate and conformal adhesion to underlying tissues, with low immune-toxicity and good biocompatibility. On clinical evaluation of the efficacy of silk fibroin for teat obstruction, a significant difference could be noticed in the healing period (in days) of animals treated with silicone implant (14.5±0.95) and electrospun silk fibroin implant (10.6±0.80) (Table 1). In group I, four cases with proper adhesion of implant to the site of application showed complete recovery without any sign of recurrence. The two cases where the implant failed to adhere resulted in recurrence of obstruction. In group II, all the cases resulted in proper adhering of implant with complete healing of wound and free flow of milk.

Table 1: Healing period (in days) of animals treated with silicone implant (group I) and electrospun silk fibroin implant (group II).

Healing period in days (Mean ±SE values)	Group I	Group II	P value	Remarks
	14.5±0.95	10.6±0.80	0.0048*	Significant

There was no significant difference in milkability of animals in both groups on day 0 and day 10, whereas a highly significant difference could be noticed on day 20 and day 30 (Table 2). Group II animals had a better recovery and return to higher milkability when compared to group I animals. Day 0 and day 10 milk samples did not show a significant difference in the somatic cell count, whereas there was a significant difference in somatic cell count between group I and group II on days 20 and 30 (Fig. 2), indicating the efficacy of biomaterial in controlling infection.

According to Catto *et al.* (2015), electrospinning (ES) was the simplest and the most efficient process for nanofiber production when compared to other common techniques like self assembly, phase separation etc. On an average, 3.56 g of dried silk

Table 2: Milkability of animals treated with silicone implant (group I) and electrospun silk fibroin implant (group II) after surgery.

Number of days	Mean±SE of milkability in mL			
	Group I	Group II	P value	Remarks
0	250.83 ±26.83	223.33 ±24.17	0.065	Not significant
10	458.33 ±24.82	493.33 ±18.19	0.095	Not significant
20	711.66 ±28.91	831.66 ±26.02	0.001	Highly significant
30	1500.83 ±39.37	1596.66 ±27.28	0.001	Highly significant

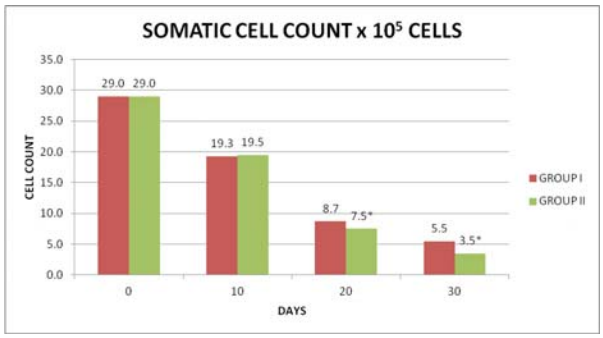

Fig. 2: Somatic cell count X 10^5 cells

fiber was obtained from initial digestion of 5 g of silk cocoon, which on further processing gave a final concentration of 8.2% silk. The porosity of the collected material is critical for its regenerative capacity. The time of spinning can alter the porosity of the collected mat. Silk fibroin: Polyethylene oxide ratio of 2:1, distance between needle tip and the collecting drum at 12 cm and flow rate of silk fibroin PEO mixture at 0.5 mL/hr were optimised to obtain the mat of 0.2 mm thickness with a pore size of 2-5 µm, over a period of 5 hr. Pore size is critical for preventing degradation of the biomaterial by collagenase and those with pore size of 5 µm or less have better withstanding capacity against collagenase (Luo *et al*., 2015)

Quick and accurate determination of cell viability could be achieved by Trypan blue assay, where more than 90% of the cells were seen viable after direct contact incubation with electrospun silk fibroin. MTT assay, provided a higher optical density, which could be noticed in the teat epithelial cells incubated with electrospun silk fibroin compared to the values obtained for normal teat epithelial cells on the respective days (Table 3) and maximum difference was noticed on 5th day. Higher the optical density, higher the metabolism of MTT

Table 3: Mean±SE of MTT assay values for biocompatibility of electrospun silk fibroin biomaterial with teat epithelial cells.

Days	Mean±SE of OD at 540 nm	
	Teat epithelial cells	Teat epithelial cells + electrospun silk sheet
3 days	1.45±0.15	1.56±0.05
5 days	1.18±0.15	1.69±0.05
7 days	1.4±0.15	1.52±0.05

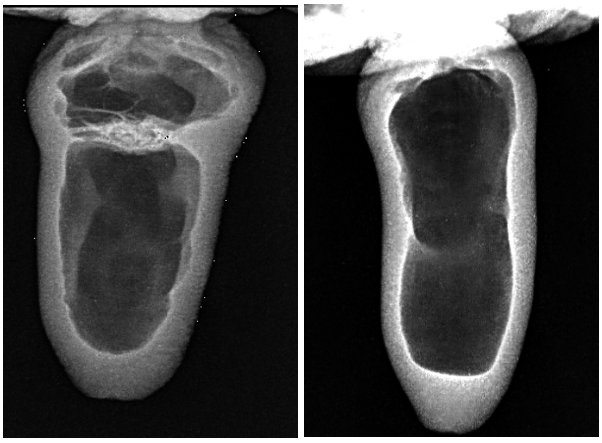

Fig. 3a: Contrast radiography of the teat with obstruction; **Fig. 3b**: Teat healing after removal of the implant.

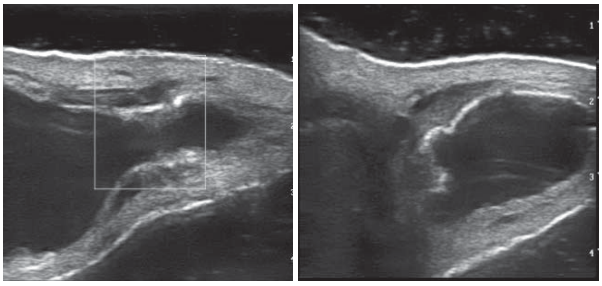

Fig. 3c: Ultrasonography of the lesion. **Fig. 3d:** Ultrasonography after removal of the implant.

and consequently, less cytotoxic is the material tested as described by Barud *et al*. (2015). In addition radiographic evaluation and ultrasonographic study of the teat before and after insertion of the implant provided information on the healing pattern (Fig. 3a, b, c and d).

From the present study, it could be concluded that silk fibroin could be used as an effective biomaterial for teat obstruction in cattle. Its slow degradability and impressive biocompatibility helped in rapid wound healing and avoided the chances of recurrence. This makes it superior to ordinary silicone teat implants.

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References

Alacam, E., Dinc, D.A., Guler, M. and Elma, E. 1990. Occurrence and radiological studies of different teat changes in milk cows. Dtsch Tierarzti wochenschr. **97**: 523-525.

Aruljothi, N., Balagopalan, T.P., Alphonse, R.M.D., Kumar, B.R. and Kumar, R. 2009. A clinical study on the use of prosthetic tubes for treatment of teatobstruction in cows. Indian J. Vet. Surg. **30**: 47-48.

Barud, H.G.O., Barud, H.S., Cavicchioli, M., Amaral, T.S., Oliveira, O.B., Santos, D.M., Petersen, A.L.O.A., Celes, F., Borges, V.M., Oliveira, C.I., Oliveira, P.F., Furtado, R.A., Tavares, D.C. and Ribeiro, S.J.L. 2015. Preparation and characterization of a bacterial cellulose/silk fibroin sponge scaffold for tissue regeneration. Carbohydrate Polymers **128**: 41-51.

Catto, V., Fare, S., Cattaneo, I., Figliuzzi, M., Alessandrino, A., Freddi, G., Remuzzi, A. and Tanzi, M.C. 2015. Small diameter electrospun silk fibroin vascular grafts: Mechanical properties, in vitro biodegradability, and in vivo biocompatibility. Mater. Sci. Eng. **54**: 101-111.

Franz, S., Floek, M. and Parisot, M. H. 2009. Ultrasonography of the bovine udder and teat. Vet. Clin. Food Anim. **25**: 669-685.

Kundu, B., Rajkhowa, R., Kundu, S.C. and Wang, X. 2013. Silk fibroin biomaterials for tissue regenerations. Adv. Drug Delivery Rev. **65**: 457-470.

Luo, Z, Zhang, Q., Shi, M., Zhang, Y., Tao, W. and Li, M. 2015. Effect of pore size on biodegradation of silk fibroin scaffolds. Adv. Material Sci. Eng. Volume 2015 | Article ID 315397 | https://doi.org/10.1155/2015/315397

Querengasser, K., Geishauser, T., Hoptner, C. and Tradat, F. 1999. Effect of teat dilators and teat canulas on udder health. Bovine Pract. **32**: 130-135.

Rockwood, D.N., Preda, R.C., Yucel, T., Wang, X., Lovett, M.L. and Kaplan, D. 2011 Material Fabrication from *Bombyx mori* silk fibroin. Nat. Protoc. **6**: 1-43. doi: 10.1038/nprot.2011.379.