Application of THEKA: Synthetic Substitute Dura, in Animal Study: Preliminary Report

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Introduction

The meninges covering the brain include the pia mater, arachnoid mater and dura mater. The dura mater is the farthest of the three layers of meninges from the brain. The dura mater is a thick, tough and fibrous-like tissue. To protect the brain, prevent infection and for CSF leakage shielding, neurosurgeons have to pay special attention to closing the dura mater tightly when finishing an operation. An incomplete dural closure is one of the most serious problems that can occur with an operation. Commonly, when finishing a brain operation, surgeons tightly close the dura mater by primary closure or dural graft with pericranium. On the other hand, if dura closure is not feasible, in some cases a dura substitute is needed. Generally, there are several types of dura substitute including autologous, allografts, xenografts and synthetic material. Autologous grafts are tissues that are donated from one part of the individual who is undergoing surgery, such as fascia lata, temporalis fascia or pericranium. Autologous grafts are the best & safest choice because they do not induce toxicity or severe inflammatory or immunologic reactions, and also do not transmit diseases, but the drawbacks are insufficient availability of the desired size and shape of donor tissue, particularly when the dural defect is large. Harvesting fascia lata requires an additional operation, so complications at the donor site can occur as well, such as wound infection, hematoma and pain. Allografts are tissues that are obtained from the same species with a different genotype donor, such as cadaveric human skin and cadaveric lyophilized dura mater. The drawbacks are immunomediated inflammatory reactions induction and disease transmission such as HIV infection, hepatitis and also fatal neurodegenerative disorder, Creutzfeldt-Jakob disease (CJD), thus human allografts were banned for further use. Xenografts are tissues taken from a different species donor. The xenograft dura substitutes that are available today are bovine or ovine pericardium, porcine intestinal sub-

mucosa (peritoneum), and collagen-based material obtained from equine achilles tendon. The disadvantages of using dura substitute, which is composed of a different species donor tissue graft, are tissue rejection and zoonotic diseases. At present there is a lack of evidence about the formulation and the results of commercialized synthetic dura substitutes. In this present study, the author would like to propose a new synthetic dural substitute based on the mixture of oxidized regenerated cellulose (ORC) and polycaprolactone (PCL). The primary objective is to fabricate a new synthetic dural substitute based on the mixture of oxidized regenerated cellulose (ORC) and polycaprolactone (PCL) and the secondary objective is to characterize the properties and effectiveness of this combination (ORC/PCL) as a novel dural substitute.

Materials and Metods

Materials

Poly (ε -caprolactone) (PCL) is a semi crystalline aliphatic polyester. It is common used as a medical biomaterial. It is gradually biodegradable by an outdoor microorganisms and hydrolytic mechanisms and is biocompatible, so it is very suitable for the design of long-term implants as a primary scaffold.

Oxidized regenerated cellulose (ORC) is a water insoluble cellulose derivative, which is prepared by the oxidation of cellulose fibers with nitrogen dioxide. This property enhances the intracellular digestion of ORC via several resident hydrolytic enzymes. So it can be absorbed in 7 to 14 days after implantation in a porcine model, and produces no foreign body reactions, so it is more suitable for the design of short-term implants as a secondary scaffold. It also possesses hemostatic and broad-spectrum antimicrobial properties.

N-methyl-2-pyrrolidone (NMP) is a slightly yellow clear liquid with low volatility and flammability. It is used as a biodegradable solvent in different fields of applications, particularly in pharmaceutical industry, and has gained Food and Drug Administration (FDA) approval.

Methods

1. Fabrication of PCL/ORC composite sheet

Polycaprolactone (PCL)/oxidized regenerated cellulose (ORC) composite was prepared by the solution infiltration process. The composite formulations were divided into 2 groups, designated P10: PCL 10 g in 100mL N-methyl-2-pyrrolidone and P20: PCL 20g in 100mL N-methyl-2-pyrrolidone. ORC sheets (Surgicel®, Ethicon Inc.) were used as a receiver in both groups by casting on the one side of the ORC sheet and drying out the solvent in the oven at 40°c.

Formulations	Polycaprolactone (PCL)	N-methyl-2-pyrrolidone
P10	10 g	100 mL
P20	20 g	100 mL

2. Dural implantation study

The study groups were separated by the types of dura substitute into 3 groups, which were pericranium as the control group, P10 and P20. The study subjects were observed for 3 periods of time which were 1, 3 and 6 months. The sample size for each group as calculated by one-way analysis of variance (ANOVA) formulation was 5 subjects. There were also 5 subjects for pilot study, which we decided to terminate in

3 months. As a result, the total number of subjects was 50. However, this preliminary study reports on only the 1-month period group along with four rabbits of the 3-month pilot subjects. As a result the total number of subjects in this preliminary study was[19].

After animal IRB approval, five 2.5-3kg, young adult, male, New Zealand white rabbits were recruited into 3 groups (Pericranium group as a control group, P10 and P20). The rabbits were all out bred from the National Laboratory Animal Centre of Thailand, Salaya, Nakhon Pathom, which met the requirements for research animals. All of them were supported at the Central Animal Facility (CAF), the Faculty of Science, Mahidol University, Bangkok under strict hygienic conventional conditions pre- and post-operatively. They were kept in suspended cages, fed by standard diet. Their health conditions were monitored daily by a veterinarian and CAF staff.

The rabbits were premedicated by the combination of Ketamine (7 mg/kg), Dextomitor (5 μg/kg) and Midazolam (0.5 mg/kg) for providing sedation and analgesia prior to anesthetic induction in order to allow endotracheal intubation. After intubation succeeded, the level of anesthesia was maintained with an induction dose of 5% Isoflurane inhalational anesthesia and followed by a maintenance dose of 3% Isoflurane, which was delivered via endotracheal tube. The levels of deep and stable anesthesia were monitored by a corneal and toe pinch reflex. The rabbits were kept warm by using heating pads throughout the operation.

The rabbits were in prone position. The scalp was shaved and prepped with Chlorhexidine under the sterile technique. The 5cm rostral-to-caudal linear incision was done at the right parietal region, caudal to the eye globe level. The 1x1cm section of pericranium was harvested in the control group. The scalps were dissected subpericranially until the skulls with the junction of the sagittal-coronal suture were identified as shown in Figure 1.

After the self-retractor was placed at the scalp layer, a high-speed electrical drill and Kerrison's rongeur were used to perform a 2x4 cm osteoclastic craniectomy at the right side of the sagittal suture and caudal to the coronal suture quadrant. Under the microscopic view, the durotomy was done by using No.24 needle and extended by hook dissector. The diameter of dural defect was about 4 mm as shown in figure 2.

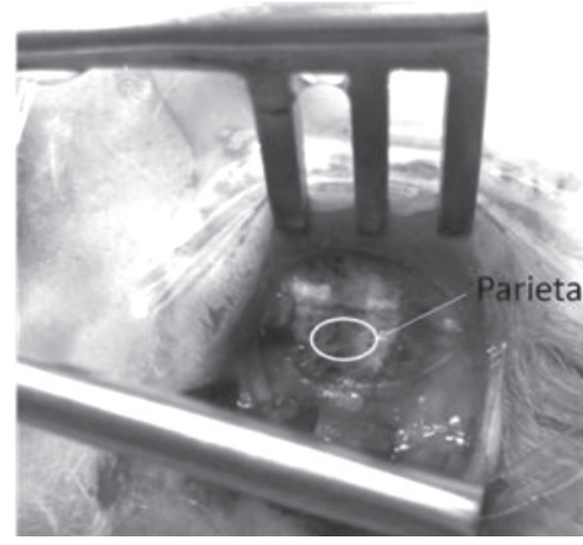

Figure 1: Area of craniectomy

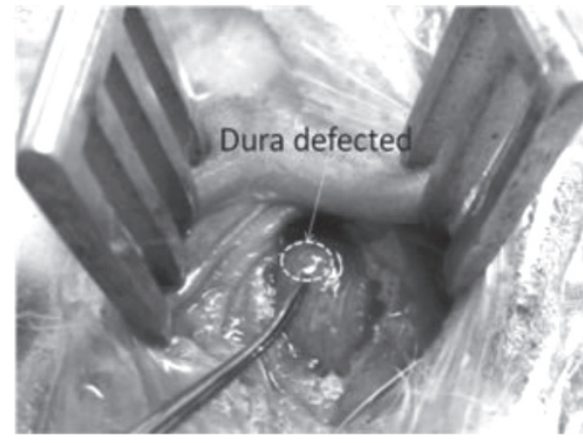

Figure 2: Durotomy site

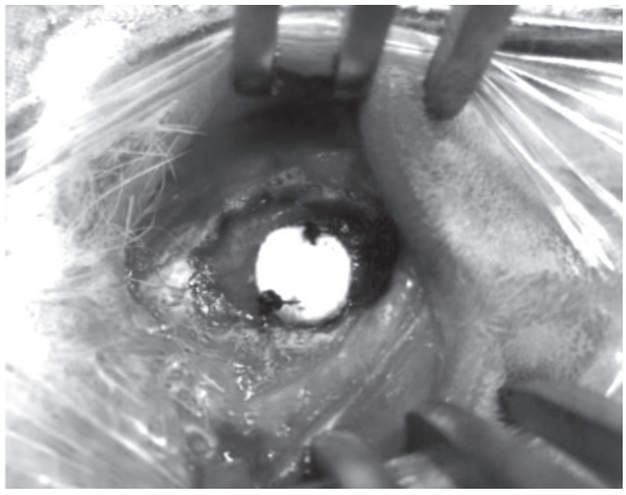

Figure 3: Dura substitute suture

The 6 mm dura substitutes would be laid to cover the dural defects. In the THEKA dura substitute groups, we designed the ORC surface to be in contact with the brain cortex surface. After that, the dural substitute was sutured with the edge of the dura defect by using 5-0 non-absorbable silk for 2-3 stitches as shown in Figure 3. Saline irrigation was done. The bleeding was checked and stopped by using Fibrillar® absorbable hemostat (Ethicon, Inc.). The scalps were closed by using 4-0 non-absorbable Nylon as shown in figure 4.

Following the operation, the rabbits were given Enrofloxacin (Baytril®) (4.0 mg/kg) via subcutaneous injection once daily for 5 consecutive days to prevent an infection, Metoclopramide (0.5 mg/kg) via subcutaneous injection once daily for 2 consecutive days to stimulate gastrointestinal tract movement and Carprofen (Rimadyl®) (4.0 mg/kg) once daily and Tramadol (2.0 mg/kg) twice daily via subcutaneous injection for 3 consecutive days to relieve the pain. The rabbits were housed in individual cages with free access to food and water. Postoperative complications were monitored by a veterinarian and CAF staff for any signs of infection and CSF leakage through the incision site.

Figure 4: Immediate postoperatively result

At the end of the each time point, the rabbits were anesthetized by Xylazine (1 mg/kg) and Zoletil (5 mg/kg) via intravenous injection to allow endotracheal intubation then maintained by 3-5% Isoflurane inhalation anesthesia via endotracheal tube. After the deep levels of anesthesia were reached, CSF was collected via cisterna magna punctures for cell differentiation, then the rabbits were euthanized by an overdose of Sodium pentobarbital (Nembutal®) (100 mg/kg) via intravenous injection. The rabbits were decapitated and degloved. The whole brain with the overlying dura and dura substitutes were preserved in neutral formalin buffer for 5 days, then skulls were decalcified by 10% EDTA immersion for 2 weeks and embedded in paraffin and microtomed for 5 μm sections for further histopathological study.

Analysis

1. Cerebrospinal fluid (CSF) examination

CSF was drawn from the cisterna magna puncture for cell differentiation to identify any inflammation or infection.

2. Microscopic histopathological study of implants

The tissue was blocked and stored in neutral formalin buffer then decalcified by 10% EDTA immersion, processed and embedded in paraffin. The samples were microtomed to 5 μm sections and stained in H&E, then assessed. The assessments were done in a semi quantitative scale and the categories of evaluation are shown in the table-1.

After the assessment scores were done in all categories, mean and standard deviation were calculated in each group then analyzed by using one-way analysis of variance (ANOVA), followed by Newman-Keuls for multiple comparisons. $P<0.05$ designated a significant different between groups.

Results

1. Cerebrospinal fluid (CSF) examination

All of the subjects (19 rabbits) showed no signs of infection or inflammation (fever, surgical site D/C, anorexia, neurological deficit, seizure) postoperatively in this study and the CSF examinations at the termination point were confirmed by showing no WBC was found in the cell differentiation.

2. Microscopic histopathological study of implants

The tissue samples were blocked and stored in neutral formalin buffer, then decalcified, processed and embedded in paraffin; after that microtomed to be 5 μm sections and finally stained in H&E. The representative of most slides of each group are as shown below.

Table 1: Scoring criteria of implant histopathology

Scoring criteria
1. Adhesion to adjacent tissue
4 Extensive adhesion
3 Moderate adhesion
2 Mild adhesion
1 Few adhesion
0 None
2. Anchorage of dura substitute to dura
4 Complete anchorage (100%)
3 > 75% anchorage
2 50-75% anchorage
1 < 50% anchorage
0 None
3. Replacement of dura substitute with host tissue
4 Complete replacement (100%)
3 > 75% replacement
2 50-75% replacement
1 < 50% replacement
0 None
4. Degree of fibrosis
4 Severe fibrosis
3 Moderate fibrosis
2 Mild fibrosis
1 Few fibrosis
0 None
5. Vascularization
4 Severe vascularization
3 Moderate vascularization
2 Mild vascularization
1 Few vascularization
0 None
6. Hemorrhage
4 Severe hemorrhage
3 Moderate hemorrhage
2 Mild hemorrhage
1 Few hemorrhage
0 None
7. PMN infiltration
4 Abundant infiltration of PMN
3 Moderate infiltration of PMN
2 Occasional infiltration of PMN
1 Few infiltration of PMN
0 None
8. Foreign body response (Macrophages or Foam cells)
4 Abundant infiltration of macrophages and giant cells
3 Moderate infiltration of macrophages and giant cells
2 Occasional infiltration of macrophages
1 Few infiltration of macrophages
0 None
9. Osteoclasts
4 Abundant osteoclasts
3 Moderate number of osteoclasts
2 Small number of osteoclasts
1 Few osteoclasts
0 None

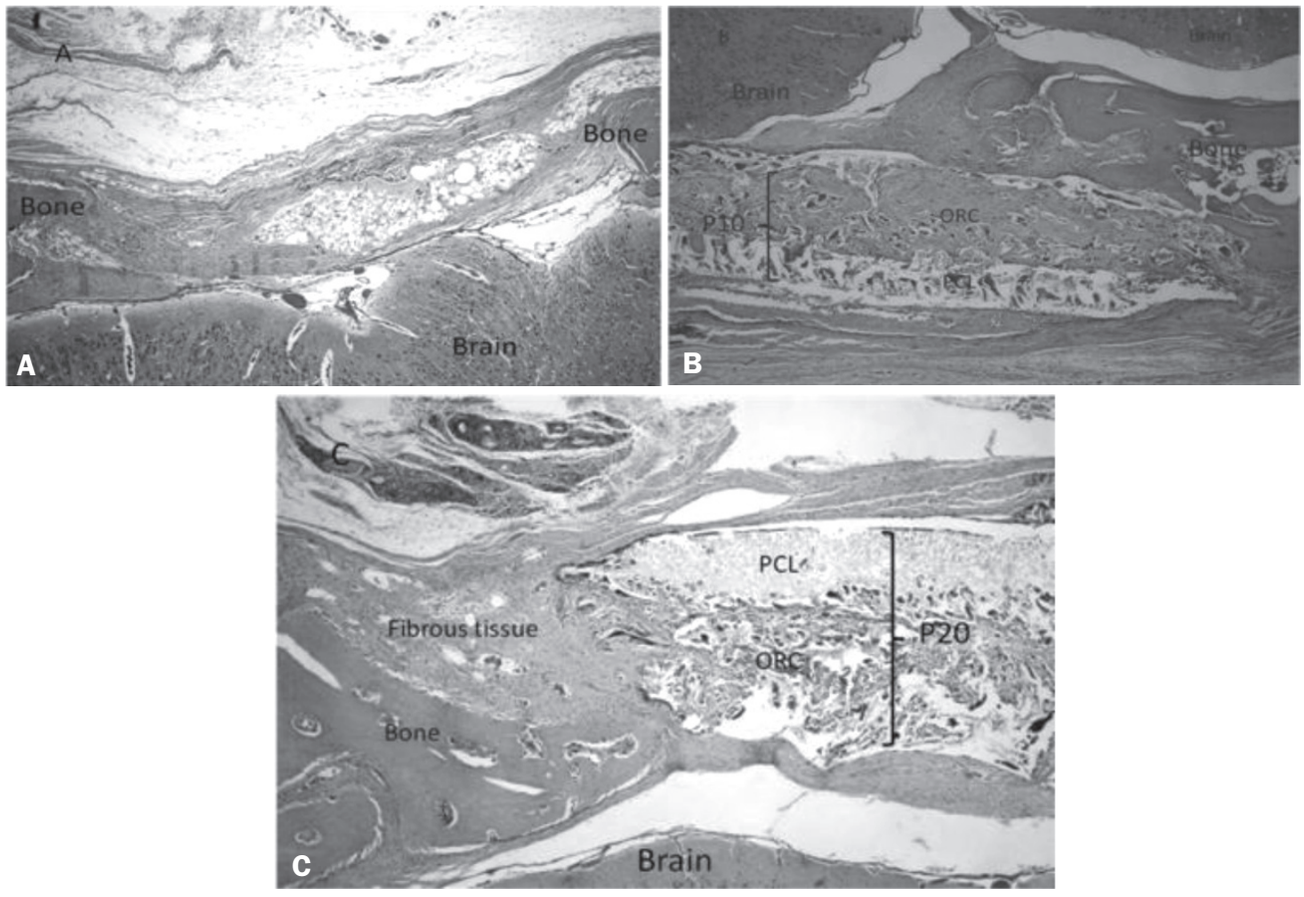

Figure 5: Microscopic examination of dura substitutes

5A: Autologous tissue from the pericranium (control) group – bone edges of craniectomy site are visible at the left & right sides and the brain cortex is seen below. Pericranium tissue could be identified in the middle between the bone edges with adipose tissue inside appearing as multiple bubbles with the fibrous tissue around it and there are slightly inflamed cells around a foreign body, which could be the silk suture, above it. There was a little bone formation as shown near the left bone edge. There was a neo-dura lining formation seen as a thin blue epithelium cells line between the fibrous tissue-brain cortex surfaces. There was also shown a minimal adhesion of the brain surface.

5B: THEKA P10 dura substitute – bone edge is shown at the right side of image and the brain cortex above. THEKA P10 is shown in the middle of the image. Because ORC was placed in contact with the brain surface and is more degradable than PCL, the ORC acted as a secondary scaffold for the neovascularization process, fibrous tissue infiltration and osteoclastic activity induction. Because PCL is less stainable and less degradable, the PCL layer shows in a loose cavity with minimal staining of proteinaceous content, which might be collagen. Neo-dura lining formation is detectable as a thin line of blue epithelium cells, but appeared to be slightly appeared less than in the autologous tissue group, and there was also new fibrous tissue near the bone edge without brain surface adhesion. There were also no active inflammatory cells such as neutrophils.

5C: THEKA P20 dura substitute – bone edge is seen at the left side of the image and the brain cortex below. THEKA P20 can be seen in the middle-to-right side of the image. The healing process was appeared to be just like the THEKA P10, with the thicker loose stained area of PCL observed, as a greater concentration of PCL made a thicker layer of PCL. There were also neo-dura lining and fibrous tissue formation apparent without brain surface adhesion or active inflammatory cells.

Table 2 summary the microscopic examination of the histopathological study

	Adhesion to adjacent tissue	Anchorage of dura substitute to dura	Replacement of dura substitute with host tissue	Degree of fibrous tissue	Vascularization	Hemorrhage	PMN infiltration	Macrophages (Foamy cells)	Osteoclasts
Autologous									
1 month									
Boonden	0	4	3	3	2	0	0	2	1
Boonroong	0	4	3	3	2	0	0	3	1
Boondong	1	3	2	2	2	0	0	1	1
Boonsak	0	4 with bone	3	2.5	2	0	0	2	1
Boonyai	0	4	2	2	2	0	0	1	1
P10									
1 month									
Boontuem	2	3 with bone	2	2	3	0	0	3	3
Boonkwan	0	4 with bone	2	3	3	0	0	2	3.5
Boonwaen	1	3 with bone	2	3	3	0	0	4	3.5
Boonploy	0	3	2	2	3	0	0	2	3.5
Boonphet	2	4	2	2	3	0	0	2	3.5
P20									
1 month									
Boonsoong	0	3.5 with bone	3	2	3	0	0	2.5	3.5
Boonsawang	0	3.5 with bone	3	2.5	3	0	0	2	4
Boonsawai	0	4 with bone	3	2	3	0	0	3	4
Boonnak	1	3 with bone	2.5	2	3	0	0	3	4
Boonnoon	1	3 with bone	2.5	2.5	2.5	0	0	1.5	4
3 months									
Autologous									
Boonrod	N	2	0	3	1	0	0	0.5	0
P10									
Boonruer	0	3	2	3	2	0	0	1	4
Boonchai	1.5	2 with bone	1.5	2	2	0	0	0.5	4
P20									
Boonmee	2.5	2	2	2.5	2	0	0	1.5	3.5

The data above were calculated and analyzed statistically. The results are shown in Table 3.

Discussion

In this present study, we would like to propose the fabrication of synthetic dura substitute (SDS) which is available to use, can be stored at the room temperature with no need to freeze, has a long shelf-life time storage and no risk of zoonotic transmitted disease. This SDS is named THEKA. It is composed of polycaprolactone (PCL) & oxidized regenerated cellulose (ORC). The PCL is the primary scaffold and the ORC is the secondary scaffold. The primary scaffold is the main structure that will maintain the strength of the regenerated cellular aggregation such as fibroblast, osteoclast and neovascularization while the secondary scaffold will create the tiny space for regenerated cellular aggregation when it is dissolved.

After the animal IRB approval, we had already tested THEKA both in vitro and in vivo. Regarding in vitro test, tensile modulus, tensile strength, breaking elongation, degradation test and cell proliferation and

Table 3

	Autologous Mean	P10 Mean	P20 Mean
Adhesion to adjacent tissue	0.2±0.45	1.0±1.00	0.4±0.55
Anchorage of dura substitute to dura	3.8±0.45	3.4±0.55	3.4±0.42
Replacement of dura substitute with host tissue	2.6±0.55	2.0±0.00	2.8±0.27##
Degree of fibrous tissue	2.5±0.50	2.4±0.55	2.2±0.27
Vascularization	2.0±0.00	3.0±0.00****	2.9±0.22***
Hemorrhage	0.0±0.00	0.0±0.00	0.0±0.00
PMN infiltration	0.0±0.00	0.0±0.00	0.0±0.00
Macrophages (Foam cells)	1.8±0.84	2.6±0.89	2.4±0.65
Osteoclasts	1.0±0.00	3.4±0.22****	3.9±0.22****, ###

Data represent the standard deviation (SD) of five animals in each group. Values were expressed as mean ± SD. * $p<0.05$, ##$p<0.01$, ***$p<0.001$, ****, #### $p<0.0001$

Statistically significant differences were determined using one-way analysis of variance (ANOVA), followed by Newman-Keuls for multiple comparisons. ***, **** Significant differences from autologous group. ##, #### Significant differences from P10 group.

cell viability had been done. Additionally, in vivo test was performed with 50 rabbits by implanting the SDS then short-term and long-term effects have been monitored for 1, 3 and 6 months in separate groups. In this present study, a preliminary report of the 1st month is reported. The compatibility of SDS in animal study has revealed the feasibility of fabrication and implanting in animals. The further study is on going for the next year and also we are looking forward to identifying the compatibility at the cellular level after 6 months of the animal implantation.

Conclusion

Synthetic dura substitute is emerging as one of the choices for dural repair and preventing CSF leakage. THEKA is an example of a synthetic dura substitute with in vitro and in vivo experimental support. Further study is needed to identify the compatibility in human model.

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