Research Report

Fibrin glue and demineralized bone matrix effect on autologus cartilage graft in microtia reconstruction

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ABSTRACT

Background: Microtia reconstruction is a challenge for ENT Head and Neck surgeons. Various surgical techniques using autograft cartilage have been done to perform auricular reconstruction. Knowledge of cartilage graft concerning resorption process that affected the size, form, and aesthetic subunit of the ear is mandatory. **Purpose:** To evaluate the success of cartilage autograft by identifying chondrocyte apoptosis, tissue degradation based on cell character, matrix homogeneity, fibrosis, proteoglycans, collagen and Transforming Growth Factor β (TGF β) expression in application of Fibrin Glue (FG) and or Demineralized Bone Matrix (DBM) after 12 weeks in microtia reconstruction by Nagata technique. **Methods:** Quasi-experiments. FG and/or DBM were applied on the rest of the 12 ear cartilage framework which was implanted on mastoid area. Apoptosis was examined by TUNEL. Safranin O staining and modified Mankin's score was used to evaluate cartilage degradation and TGF β expression by ELISA. **Results:** FG or DBM on cartilage graft showed significant increase in chondrocyte viability compare with control group (p=0.00). Minimal fibrosis, more homogeneous extracellular matrix, decreased proteoglycan and minimal thickening of collagen, had significant differences compared with control or FG-DBM group. Structure differences occurred among cartilage graft after 12 week implantation whereas FG showed minimal fibrous tissue, normal cell character, proteoglycan, collagen, and tissue homogeneity ($p< 0.05$). **Conclusion:** FG is highly recommended to reduce degradation of cartilage graft in microtia reconstruction. DBM can be still used to maintain chondrocyte viability, proteoglycans, and collagen.

Keywords: cartilage graft, fibrin glue, demineralized bone matrix, transforming growth factor β, Mankin score.

ABSTRAK

Latar belakang: *Rekonstruksi mikrotia merupakan tantangan bagi ahli bedah THT-KL. Berbagai teknik operasi menggunakan rangka telinga dengan tandur kartilago autologus telah dilakukan untuk rekonstruksi mikrotia. Pengetahuan mengenai tandur kartilago sangat diperlukan, mengingat tandur dapat mengalami resorpsi dengan berjalannya waktu, sehingga mempengaruhi ukuran, bentuk, dan detil estetik subunit daun telinga.* ***Tujuan:*** *Mengetahui viabilitas kondrosit, degradasi jaringan berdasarkan perubahan karakter kondrosit, fibrosis, homogenitas matriks, ekspresi proteoglikan dan kolagen serta ekspresi transforming growth factor β (TGF β) dengan atau tanpa fibrin glue (FG) dan/atau demineralized bone matrix (DBM) pada rekonstruksi mikrotia setelah 12 minggu penanduran.* ***Metode:*** *Quasi-eksperimen. FG dan/atau DBM digunakan pada sisa tandur autologus kartilago rangka telinga, dilanjutkan pemeriksaan apoptosis dengan TUNEL. Pewarnaan Safranin O untuk menilai degradasi jaringan dengan skor modifikasi Mankin dan ekspresi TGF β dengan ELISA.* ***Hasil:*** *penambahan FG atau DBM pada tandur kartilago, viabilitas sel meningkat berbeda bermakna dengan tanpa perlakuan atau FG-DBM.(p=0.00), fibrosis minimal, matriks lebih homogen, penurunan proteoglikan dan penebalan kolagen minimal berbeda bermakna dengan kelompok tanpa perlakuan dan campuran FG-DBM. Terjadi perbedaan struktur jaringan setelah 12 minggu, FG mempunyai nilai fibrosis yang terendah, karakter sel normal, proteoglikan, kolagen, dan homogenitas jaringan (p<0,05).* ***Kesimpulan:*** *Penggunaan FG sangat dianjurkan untuk mengurangi degradasi tandur kartilago autologus pada rekonstruksi mikrotia.*

Dalam keadaan tidak memungkinkan dapat digunakan DBM karena masih baik dalam mempertahankan viabilitas kondrosit, proteoglikan dan kolagen.

Kata kunci: *tandur kartilago, fibrin glue, demineralized bone matrix (DBM), transforming growth factor β (TGF β), Mankin score.*

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This article have presented in the 11th International Facial Plastic Surgery Symposium, June 2, 2014, New York

INTRODUCTION

In the last decade, Department of Otolaryngology- Head and Neck Surgery, Faculty of Medicine University Indonesia/ Cipto Mangunkusumo Hospital has been performing microtia reconstruction with various degree anomaly. On reconstruction procedure, ear framework is made of autologous rib cartilage graft.[1-3] The surgery procedure consists of 2 phases. The first phase is to form an ear frame continued with grafting into the subcutaneous ear region.[4] Ear pattern is formed according to the anatomy, height and width of normal ear. Sixth-seventh rib cartilage is carved in detail using knife or drill in accordance to the normal ear shape.[5-6] The second phase is performed after 12 weeks by elevating the auricle and if necessary, accompanied with auditory canal reconstruction.[7]

Cutting and carving of cartilage will arouse immunologic response and protection of cartilage matrix.[1,3,8,9] Response against trauma will stimulate cytokine production and growth factor, leading to the metabolic change of chondrocyte and matrix. Total chrondrocyte will be reduced, cell cluster will be established and there will be a change of matrix distribution and composition. Simultaneously there will be cytokine and catabolic enzyme synthesis, proteoglican loss, and type II collagen upon cartilage surface. In the subsequent process, there will be a matrix degradation exceeding synthesis causing loss of protein matrix expression.[10]

Similar response also may occur when cartilage ear frame work being implanted in mastoid area as the recipient bed. The process occurs against autologous cartilage graft or local tissue. The cartilage tissue degradation caused by the process related with the chondrocyte viability, can be evaluated from percentage of cell-experienced apoptosis.

According to Langer and Vacanti[11] tissue-engineered cartilage is the application science of tissue engineering and developed into biological material to repair and change tissue function for reconstruction purpose. The technology uses biocompatible, biodegradation, synthetic or polymer tissues as scaffold. The tissue engineering may accelerate cartilage development to be mature to its morphological character, using media in order that cell growth environment becomes stable.[6, 11,12] The technique can be used in clinical application of maxillofacial reconstruction surgery.[13]

Vacanti et al. quoted from Britt and Park[6] reported the success of tissue engineering to produce cartilage ear frame work by increasing cell viability and matrix structure to form ear frame template, which the viability of chondrocyte have been maintained by adding fibrin or growth factor.

Transforming growth factor-beta, (TGF β) is the protein produced by cells with specific activity against target cells. It is expressed during bone fracture healing process and also used for cartilage or bone defect therapy which is being a required component in tissue engineering technique.[13] By adding TGF β invitro, viability cell and matrix production can be maintained. On

clinical application of TGF β effect can be used as the therapy.[14] The use of TGF β family is demineralized bone matrix (DBM) which is a homologous graft with osteoconductive activity. DBM is made through bone extraction by eliminating mineral component and maintaining collagen and non-collagen protein including TGF β.

Fibrin glue or tissue adhesive contains fibrinogen, in nature coagulation factor XIII is substance which may strengthen tissue contact and inhibit tissue leakage. Fibrin glue (FG) may also play a role as the conductive substance of growth factor into the targeted delivery system. Cell culture and histological evaluation shows that FG does even possibly stimulate the healing process. In clinical application, FG has been used in maxillofacial reconstruction surgery including those using flap and graft.[15]

Purpose of the research was to evaluate and identify the success of autologous cartilage graft with and without FG/DBM which was assesed from chrondocyte viability, hystomorphological change and cartilage degradation process after 12 weeks of grafting.

METHODS

This quasi experiment study was approved by the Institutional Review Board of Medical Faculty University of Indonesia/Cipto Mangunkusumo Hospital Jakarta, Indonesia. We evaluated the result of cartilage graft using FG and/or DBM as intervention group in 12 microtic ear which underwent reconstructive surgery between April 2008 and August 2009 at Plastics Reconstructive Division, Department of Oto laryngology,Ciptomangunkusumo Hospital.

Surgical procedure

Auricle reconstruction was performed by harvesting 6th-7th rib cartilage graft, then shaped and adjusted to the planned ear shape. Helix is the highest part of aesthetic unit while fossa triangularis, schapa and concha are parts without cartilage. Carved cartilage was then added with FG (Beriplast® ZLB Behring GmbH Marburg, Alemania) and implanted into a skinpocket under ear region. The remaining cartilages which were intervened with DBM, FG-DBM and control group then inserted posterior to the ear framework. All this piece of cartilage will be used as buttress in second phase surgery.

After 12 weeks, ear projection was performed starting with 1 cm curved incision making a subcutaneous pocket. Fibrotic capsule was maintained for covering the ear framework. External ear canal was then created and cartilage buttress placed on the mastoid area. Retro auricular fascia flap was undermined to cover ear buttress. Finally, the posterior auricular region was veiled by split thickness skin graft.

Cartilage tissue samples were examined for biological characteristic of chondrocyte apoptosis and viabilty, fibrosis, tissue homogenity, collagen, proteoglycan and TGFβ expression. Hystomorphology, Safranin O staining, TUNEL and Elisa were carried out in Department of Biology Molecular Eijkman Institute Jakarta and Department of Pathology Anatomy, Faculty of Medicine Universitas Indonesia.

RESULTS

Evaluation of chondrocyte

The statistical analysis used pair t-test for the asessment of apoptosis, living cells, cell density between phase1-phase2 and among the intervention groups (FG, DBM, and FG-DBM and control group). Number of apoptotic cells decreased in all groups ($p<0.001$), accompanied by increased living cells after grafting $p<0.001$). However, the apoptosis in FG group was decreased from

48.84 to 19.83 and number of living cells increased from 51.16 to 79.23. before and after grafting. There were no difference of cell density and TGFβ in all groups (p>0.05).

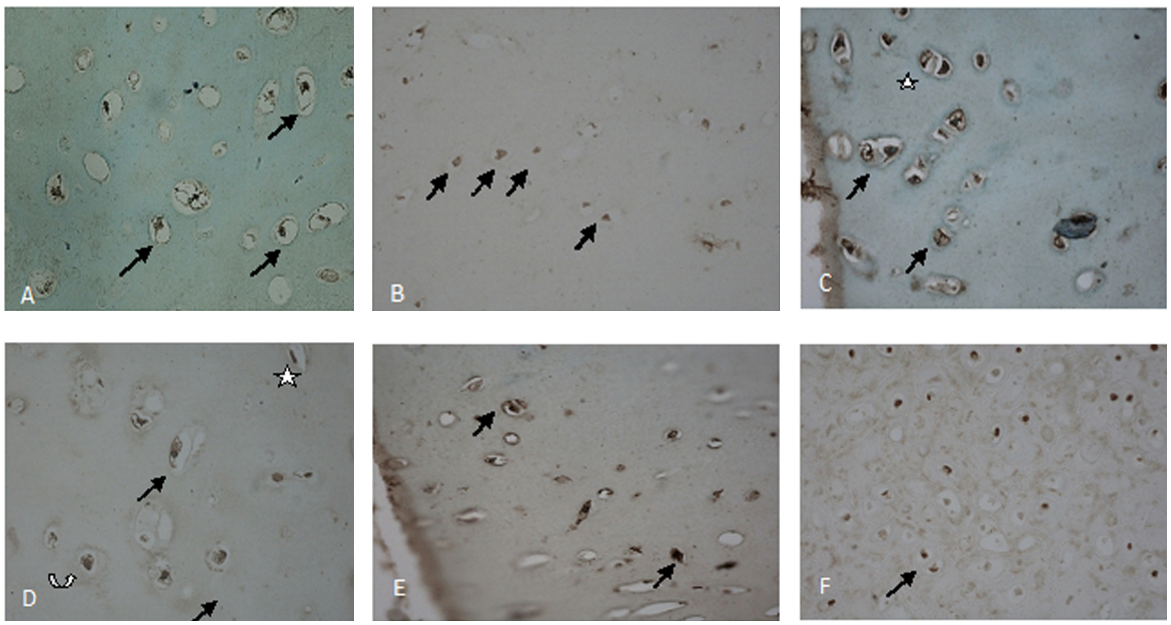
In the DBM group, number of apoptotic cell decreased from 48.84 to 27.03 (p=0.018). There was no statistically different between DBM and FG groups (p=0.592), however significant different found in apoptosis decrease of FG-DBM group. (p=0.008).

Table 1. TUNEL examination

Variabel	N(12)	Mean	SD	Min	P25	Median	P75	Max	P*	P ¶	P∃	P⊥
Apoptotic	Phasel	3,54	3,24	0	1,84	2,29	5,04	10,74				
cells (%)	Control	48,84	21,09	24,70	33,96	42,49	62,39	100	**0,000**			
	FG	19,83	11,61	3,79	12,34	16,58	29,17	42,85	**0,000**	**0,001**		
	DBM	27,03	26,89	4,17	10,77	15,47	42,44	100	**0,000**	**0,018**	0,592	
	FG-DBM	36,57	22,27	15,38	20,6	33,97	40,89	100	**0,000**	0,092	**0,008**	0,123
Living	Phasel	96,46	3,24	89,26	94,96	97,72	98,17	100				
cells (%)	Control	51,16	21,09	0	37,62	57,52	66,05	75,3	**0,001**			
	FG	79,23	11,39	57,15	70,46	82,54	87,38	96,21	**0,001**	**0,001**		
	DBM	72,97	26,89	0	57,56	84,53	89,23	95,83	**0,012**	**0,018**	0,592	
	FG-DBM	63,43	22,27	0	59,11	66,04	79,4	84,62	**0,001**	**0,092**	**0,008**	0,123
Density	Phasel	128	39	64	103	116	98,17	193				
(cell/view)	Control	140	90	0	88	129	66,05	351	0,618			
	FG	128	26	92	107	129	87,38	175	0,638	0,575		
	DBM	118	63	0	84	110	89,23	223	0,755	0,336	0,656	
	FG-DBM	116	65	0	85	107	79,4	225	0,894	0,074	0,374	0,646

P*Pair t test apoptosis, living cells among phase 1-phase 2, phase 1- FG, phase 1-DBM, and phase 1-FG-DBM
¶ Pair t test apoptosis , living cells among phase 2, FG, DBM, and FG-DBM
∃ Pair t test apoptosis, living cell s among FG, DBM, and FG-DBM
⊥ T test apoptosis in pair, living cells between DBM and FG-DBM

Wilcoxon test on cell density among phase1-phase2 and among phase2, FG,DBM,FGDBM, FG, DBM and FGDBM



Picture 1. A) Chondrocyte of rib cartilage before grafting (phase1) = living cell, with green nucleus. B) Autologous graft after 12 weeks grafting (phase 2) C) Autologous graft with FG, 12 weeks of grafting. Living chondrocyte with green nucleus (↗) apoptotic cell with brown nucleus (↗) D) Living hipertrophic chondrocyte. D) FG-DBM intervention shows chondrocyte apoptosis

Evaluation on cartilage degradation using Safranin O and modified Mankin criteria

The paramaters used in the modified Mankin criteria consist of cell character (0-3), fibrosis (0-3), loss of safranin (0-3), tissue homogenity (0-1) and collagen (0-2). Maximal total score was 12. The results were categorized into 0: Normal, 1-4: Mild degradation, 5-8: moderate degradation, 9-12: severe degradation.

The evaluation of cell scores among the treatment group shows that the FG group had the best results with the lowest score (0.67) while the DBM treated group had the score of 1.25. The FG treated also shows the best score for tissue structure 0.50 compared to DBM 0.75. The loss of safranin O stain indicated by the decrease of proteoglycan expression. The use of FG provided the best score (0.75) while DBM score was 1.17. The evaluation score of tissue integrity, in FG group was 0.58 and DBM group was 0,92 . The collagen score in the FG group was 0.42. The FG administration prevented the occurrence of tissue degradation with the best score of 2.92, followed by the DBM with score 4.75. In the control group, the average cell character score was 1.33, fibrosis 1.25, matrix homogenity 1.08 and the total score was 7.25 which showed equal with the FG-DBM score. Safranin staining in the FG-DBM group showed that cell character, fibrosis, proteoglycan, matrix homogenity and collagen showed the worst damage of extra cellular matrix, as those of the control group.

There were statistically significant differences on proteoglican, collagen and total scores between the control and DBM groups. However there was no significant difference in total scores regarding cell character, fibrosis and tissue homogenity between FG and DBM groups.

Resorption analysis based on the tissue degradation in the FG group was different from the other groups. The least resorption was in the FG group and it was statistically significant different from DBM and FG-DBM groups (<0.002). The DBM, FG-DBM and control groups which showed moderate

Table 2. Inter-groups evaluation in cell character, tissue structure, tissue integrity, proteoglycan and collagen

Parameter		FG	DBM	FG-DBM
Cell character	Phase2 without treatment	**0.005**	0.773	**0.046**
	Phase2+FG		0.083	**0.006**
	Phase2+DBM			**0.030**
Tissue structure	Phase2 without treatment	**0.021**	0.084	0.124
	Phase2+FG		0.317	**0.011**
	Phase2+DBM			**0.010**
Loss of safranin	Phase2 without treatment	**0.006**	**0.024**	0.317
	Phase2+FG		0.096	**0.010**
	Phase2+DBM			**0.020**
Tissue integrity	Phase2 without treatment	**0.034**	0.157	0.317
	Phase2+FG		0.157	**0.025**
	Phase2+DBM			0.564
Collagen	Phase2 without treatment	**0.002**	**0.010**	0.129
	Phase2+FG		0.180	**0.015**
	Phase2+DBM			0.083
Total scores	Phase2 without treatment	**0.002**	0.005	0.393
	Phase2+FG		0.036	**0.002**
	Phase2+DBM			**0.003**

Wilcoxon's non-parametric in pairs

resorption were statistically significant different from the FG group regarding tissue degradation.

The decrease of TGF β expression in various treatments showed no statistically significant difference between phase 1 and phase 2, 26.91pg/mg to 24,13 pg/mg. FG intervention elevated TGF β level from 26.91 to 31.90 pg/mg (no significant difference).

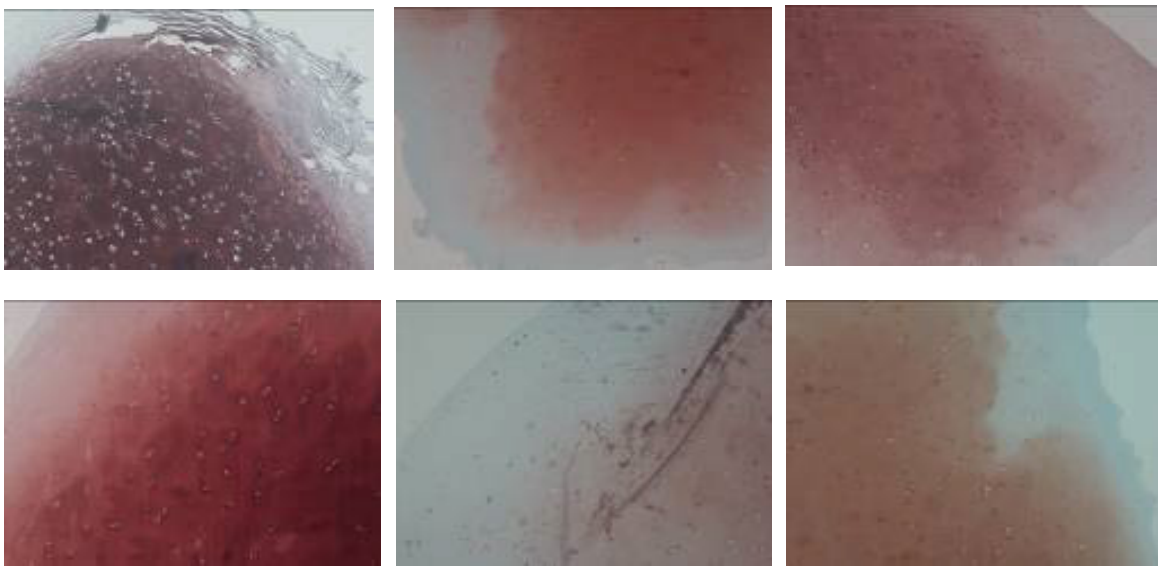
TGF β expression in DBM group showed significant decreased from 26.91 to 12.70 pg/mg (p = 0.009; CI 6.14-19.26) ,whereas there was no statistically different decrease TGF β expression in FG-DBM intervention showed no significant decrease compared with control group; but it was found significant different from FG intervention group (p=0,013; CI 12,82-27,35).

By Spearman correlation test, a moderate correlation (r=0,70) was found between apoptosis and TGF β expression in control group. Weak correlations were found between apoptosis and TGF β expresion in all

Table 3. Cartilage degradation scores

Group	Cartilage degradation			Total
	Mild 1-4	Moderate 5-8	Severe 9-12	
Control	0 (0%)	10 (83.3%)	2 (16.7%)	12 (100%)
FG	11 (91.7%)	1 (8.3%)	0 (0%)	12 (100%)
DBM	4 (33.3%)	8 (66.7%)	0 (0%)	12 (100%)
FG-DBM	0 (0%)	8 (66.7%)	4 (33.3%)	12 (100%)
Total	15 (31.3%)	27 (56.3%)	6 (12.5%)	48 (100%)

Control – FG, p<0.002 (Wilcoxon test)
Control– DBM, p=0.034 (Wilcoxon test)
Ccontrol-FG-DBM, p=0.317 (Wilcoxon test)
FG-DBM, p=0.008 (Wilcoxon test)
FG-FG-DBM, p=0.002 (Wilcoxon test)
DBM-FG-DBM, p=0.005 (Wilcoxon test)



Picture 2. A). Mankin score, total score as base line parameter. B.)Total score control group 7. Fibrosis 1, cell character 1, proteoglycan 2, homogenity 1, collagen 2,) C.) FG total score 2.(fibrosis 0, cell character 0, proteoglycan 1, homogenity 0, collagen 1,) D.) DBM Total score 5(Fibrosis 1, cell characyter 2, proteoglycan 1, homogenity 1, collagen 0,) . E). FG-DBM Total score 7. Fibrosis 1, cell characyter 1, proteoglycan 2, homogenity 1, collagen 2

intervention groups .

Resorption was evaluate among intervention group to FG-DBM groups. (<0.002). Mild resorption occured in FG group followed by DBM group. FG-DBM and control group showed moderate and severe resorption. There were significant differences in FG and DBM compare to FG-DB control group.

TGF β level in various interventions showed in table 5.8. TGF β level (tissue pg/mg) was found in all tissue degradation between phase 1 and phase 2 from 26.91 into 24, 13 tissue pg/mg (not significant). FG group showed increased of TGF β level from 26.91 into 31.90 tissue pg/mg (not significant).

Table 4. TGF β level (tissue pg/mg) before and after 12 weeks grafting

Variable	Average pg/mg tissue	SD	95% CI	p*	p¶	p∃	p⊥
Phase 1	**26.91**	**18.16**	**15.37-38.45**				
Phase 2							
Control	**24.13**	**16.31**	**13.76-34.49**	**0.320**			
FG	**31.90**	**22.23**	**6.14-19.26**	**0.787**	**0.919**		
DBM	**12.70**	**10.33**	**6.14-19.26**	**0.009**	**0.013**	**0.011**	
FG-DBM	**20.08**	**11.43**	**12.82-27.35**	**0.055**	**0.204**	**0.013**	**0.936**

* paired t test TGF β level between phase 1 and phase2

¶ t test of TGF β level among phase 2, FG, DBM, and FG-DBM

∃ t test of TGF β level between FG, DBM and FG-DBM

⊥ t test of TGF β level between DBM and FG-DBM

DISCUSSION

FG plays a role as conductive substance of growth factor to the targeted delivery system. It served as scaffold which is an application of tissue engineering, so that cell viability and quality of extra cellular matrix can be maintained.[16,17]

TUNEL on control group showed increase of apoptosis into 48.48%+/-21.1 after 12 weeks. FG group showed decrease of apoptosis into 19.83% +/-11.61. It might be possibly caused by the existence of FG as scaffold. FG will cause attachment between graft and skin and other tissues. Such as in physiological injury therapy, it will produced fibroblast in proliferation phase. During that time, various growth factors will be produced.

FG (Beriplast®) is available in local market or, as an option we can use autologous fibrin glue. The commercial preparation consists of human plasma protein (fibrinogen, active factor XIII, thrombine, apopthronine and calcium chloride). In culture cell, FG can be used to increase proliferation of chondrocyte, and extra cell matrix such as proteoglycan and collagen 4-8 times after the 14th day.[18]

In clinical experience, a reconstructive surgeon can use FG mixed with a piece of bone or cartilage, which filled into the cartilage defect as chondrocyte conductive material during the healing process.[16]

This study confirmed that FG is useful as chondrocyte inductor and was able to increase cell proliferation and matrix regeneration in facilitating cartilage repair.[19]

In DBM there is a bone morphogenetic protein (BMP), which is a derivate of TGF β1 superfamily. It is a protein expressed by

chondrocytes and shows specific activity to the target cells. This growth factor is expressed during recovery process and used in the management of cartilage or bone defect. It has been proven in vitro that by adding transforming growth factor (TGF β), cell viability and activity could be maintained, and matrix production increased. In this study, TGF β1 expression has been proven that it could prevent the occurence of cartilage graft degradation.[20,21]

Collagen expression and excessive fibrous tissue due to cartilage damage have correlation with clinical finding after surgery. Subcutaneous tissue thickening occurred and covered detailed ear frame silhouette ear sub unit. For this condition Siegert[22,23] proposed corrective subunit auricle surgery.

Fibrin Glue is highly recommended to be used in microtia reconstruction in order to increase chondrocyte viability with minimal tissue degradation of autologous cartilage graft. It correlates with increased TGF β expression. DBM can still be used alternative to maintain chondrocyte viability, proteoglycans, and collagen.

Acknowledgements

Authors are grateful to:

Dr. Endang Ch. Mangunkusumo, SpTHT-KL(K), Department of Otorhinolaryngology Head and Neck Surgery, Faculty of Medicine Universitas Indonesia/Dr. Cipto Mangunkusumo Hospital Jakarta

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