The British Association of Plastic Surgeons (2004) **57**, 423–428

ELSEVIER

BJPS
An International Journal of Surgical Reconstruction
www.bjps.com

Effects of human amniotic fluid on cartilage regeneration from free perichondrial grafts in rabbits

Güzin Yeşim Özgenel*, Gülaydan Filiz, Mesut Özcan

Department of Plastic, Reconstructive, and Aesthetic Surgery, Medical Faculty of Uludağ University, 16059 Görükle, Bursa, Turkey

Received 19 February 2003; accepted 17 December 2003

KEYWORDS
Human amniotic fluid;
Perichondrium;
Neochondrogenesis;
Hyaluronic acid; Growth factors

Summary After the chondrogenic potential of free grafts of perichondrium was shown in several experimental studies, perichondrium has been used to reconstruct cartilage tissue in various clinical situations. This study investigates the effects of human amniotic fluid on neochondrogenesis from free perichondrial grafts in a rabbit model. Since this fluid contains high concentrations of hyaluronic acid, hyaluronic acid-stimulating activator, growth factors, and extracellular matrix precursors during the second trimester, it may have a stimulating effect on neochondrogenesis.

Perichondrial grafts, measuring 20×20 mm^2 were obtained from the ears of 144 New Zealand young rabbits and were sutured over the paravertebral muscles. The rabbits were randomly divided into three groups with 48 rabbits per group. In group 1, 0.3 ml human amniotic fluid, and in group 2, 0.3 ml saline were injected underneath the perichondrial grafts. Group 3 formed the control group in which no treatment was given.

Histologically, neochondrogenesis was evaluated in terms of cellular form and graft thickness at 2, 4, 6, and 8 weeks after surgery. In group 1, the mature cartilage was generated quickly and the cartilage plate in this group was significantly thick and extensive when compared with groups 2 and 3 at 8 weeks ($p < 0.05$, ANOVA).

In conclusion, our study shows that human amniotic fluid enhances neochondrogenesis from free perichondrial grafts. The rich content of hyaluronic acid and growth factors possibly participate in this result.

Perichondrium is known to have a chondrogenic potential. Clinically and experimentally, free perichondrial grafts have been used to reconstruct cartilage in ear, larynx, nasal septum and articular cartilage defects.[1–13]

Recently, there has been interest in examining factors which may promote cartilaginous differentiation from perichondrium. In vitro studies have shown that hyaluronic acid (HA), glycosaminoglycan, exerts a regulatory function on chondrocyte activity by increasing proteoglycan synthesis and secretion.[13–17] On the other hand, HA itself is known to reduce scar formation by inhibiting lymphocyte migration, proliferation and chemotaxis, granulocyte phagocytosis and degranulation, and macrophage motility.[18–20] HA may therefore

*Corresponding author. Tel.: +90-224-4428193; fax: +90-224-4428079.
E-mail address: gozgenel@yahoo.com

S0007-1226/$ - see front matter © 2004 Published by Elsevier Ltd on behalf of The British Association of Plastic Surgeons.
doi:10.1016/j.bjps.2003.12.021

provide a mesenchymal signal for healing by regeneration rather than by scarring and fibrosis. Human amniotic fluid (HAF) obtained by amniocentesis performed in connection with prenatal diagnosis at 16–24 weeks of gestation contains high molecular weight of HA and HA-stimulating activator (HASA) in high concentrations.[21,22] It was shown that HASA stimulates wound healing by increasing the production of endogenous HA in the application region.[22–24]Hence, HA in HAF may enhance neochondrogenesis from the free perichondrial grafts.

HAF is known to be rich in potent growth factors such as epidermal growth factor (EGF), fibroblast growth factors (FGF), insulin-like growth factors (IGF) and extracellular macromolecules such as fibronectin and laminin which are known to have a growth-promoting effect on cartilage.[25–29] Thus, these factors in HAF may have a stimulating effect on the development of cartilage formation from the perichondrium.

We have previously shown that HAF enhances the new cartilage formation from rabbit ear perichondrial flaps.[30] Because of the fact that free perichondrial grafts are widely used for reconstructive purposes, in this study, we investigated the effects of HAF on neochondrogenesis from free perichondrial grafts in a rabbit model.

Materials and methods

In this experimental study, we used 144 New Zealand rabbits, with a mean age of 2 months, weighing between 900 and 1100 g. The young rabbit was selected as our experimental animal because the large ears serve as a plentiful source of perichondrium. All animal experimental procedures were approved by the Committee of Ethics on Animal Experiments in the Faculty of Medicine, Uludağ University, Bursa, Turkey. The rabbits were anesthetised with intramuscular xylazine hydrochloride [ROMPUN] 5 mg/kg body weight and ketamine hydrochloride [KETALAR] 35 mg/kg body weight, supplemented with doses of 0.1 ml ketamine hydrochloride intramuscularly when required. A single intramuscular injection of Cefuroxime, 30 mg/kg body weight, was given intramuscularly before surgery for prophylaxis.

The rabbits were randomly divided into three groups with 12 rabbits per group at each time point of 2, 4, 6 and 8 weeks after the operation.

Surgical procedure

Ears of all rabbits were operated on under sterile conditions. In the hollow of the ear, a superficial incision was made through the skin, care being taken not to enter the perichondrium. After the skin was bluntly raised, a standard area of perichondrium, $20 \times 20\ mm^2$, was incised and dissected off its' cartilaginous bed with the aid of a small periosteal elevator. The skin incisions were closed with interrupted sutures using 5/0 braided polyglactin [DEXON]. The perichondrial grafts were sutured over the paravertebral muscles of the rabbits using 6/0 monofilament polypropylene [PROLENE] sutures in a stretched position but without tension. The active surface of the perichondrium that was adjacent to the ear cartilage was transplanted to face the paravertebral muscles. In group 1, 0.3 ml HAF, and in group 2, 0.3 ml saline was injected underneath the perichondrial grafts. In group 3 (control group), no treatment was given. Skin incisions of the recipient sites were closed by 5/0 braided polyglactin sutures [DEXON].

All operations were done by the same surgeon to guarantee the complete homogeneity of the results.

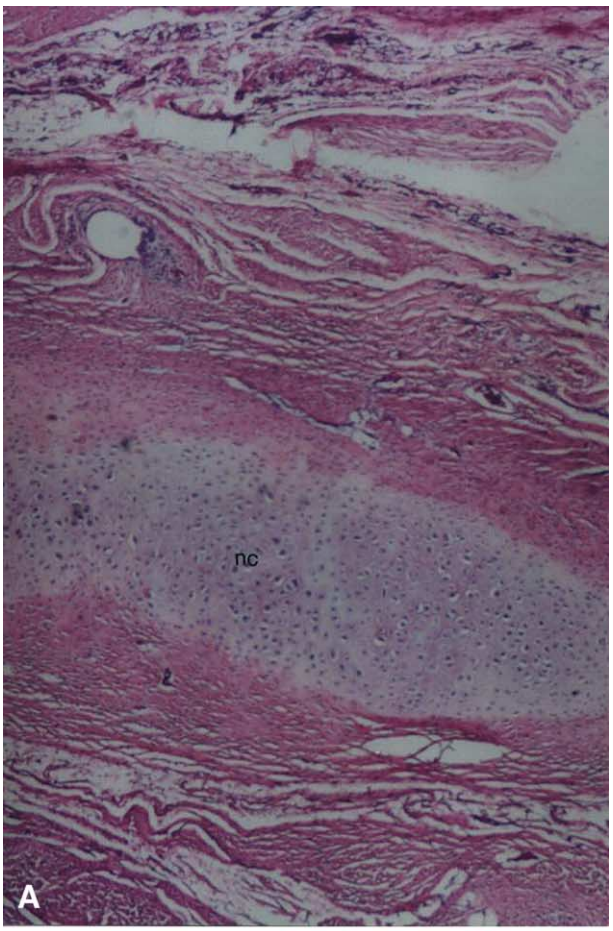
Human amniotic fluid collection

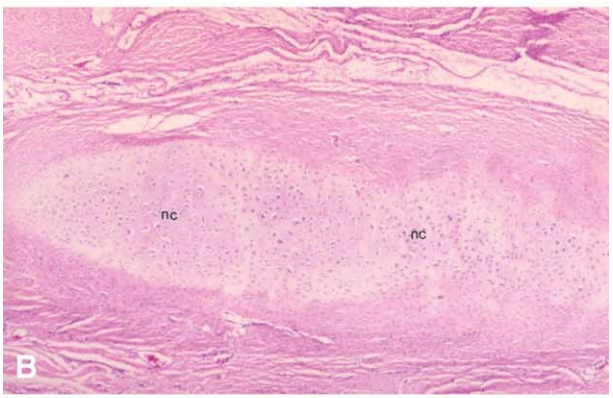
HAF was provided by the prenatal diagnosis unit from routine diagnostic amniocentesis for 16–24 gestation. Oral consent was given for each collection. The fluid was stored at $-20\ ^{\circ}C$ prior to use. The interval from collection to topical use was less than 1 week.

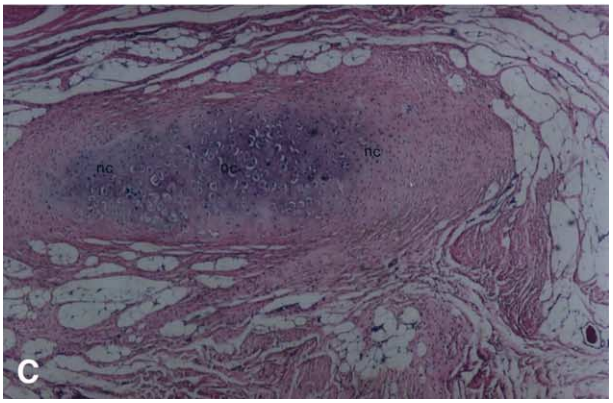
Histologic evaluation

The histological progression of neochondrogenesis from the free perichondrial grafts were evaluated at 2, 4, 6 and 8 weeks after surgery. All animals were sacrificed, and the specimens, which included the operative sites were excised en bloc. They were fixed in 10% neutral-buffered formalin and embedded in paraffin. Longitudinal serial sections 10 μm thick were cut and stained with haematoxylin and eosin and masson trichrome. All sections were studied under a light microscope. The tissue derived from the perichondrial graft was evaluated in terms of cartilage thickness and cellular form. To obtain a mean value, the thickness of neocartilage in every case was measured at 10 randomly selected points using an ocular micrometer (Periplan $6.3 \times M$, Leitz Wetzler, Germany) at $\times 400$ magnification.

Histologic sections were evaluated by a pathologist, who received no prior information of the treatment each rabbit received.







Statistical evaluation

Statistical significance in the results of neocartilage formation was determined using the one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test. A value of $p < 0.05$ was considered significant.

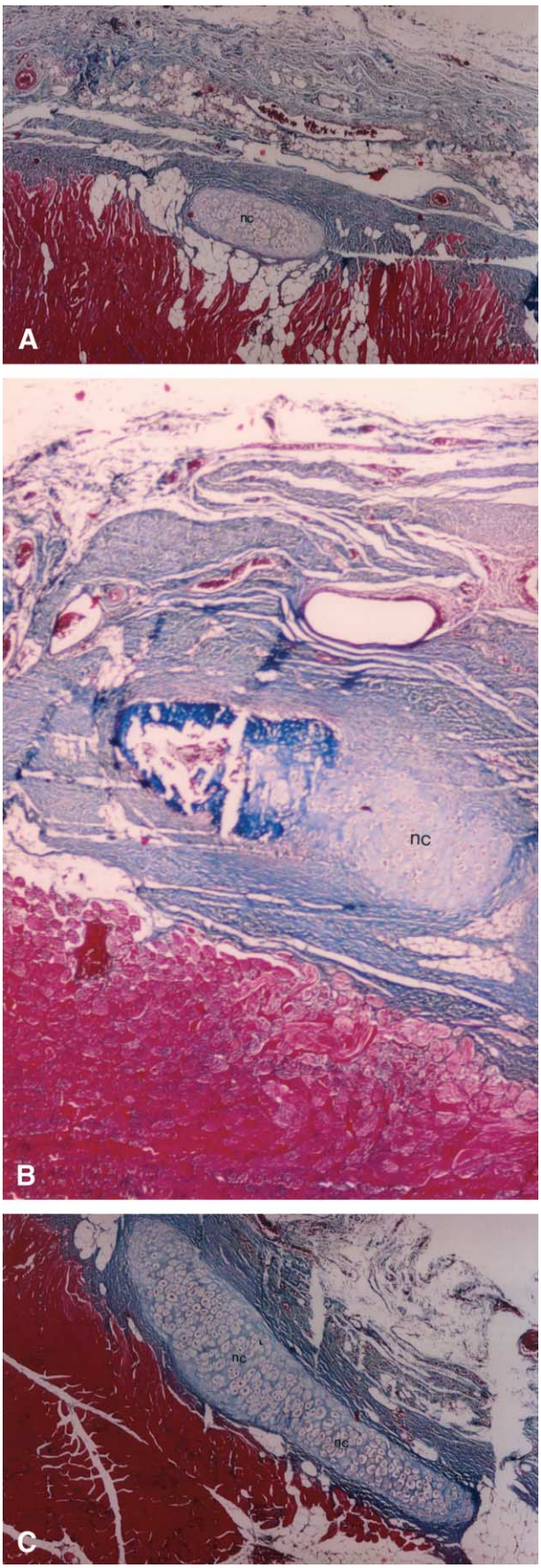
Results

Histologic evaluation

No sign of infection or inflammatory reaction was observed in any of the groups. At 2 weeks, in groups 2 (saline-treated group) and 3 (control group), no areas of neocartilage were observed, whereas, in group 1 (HAF-treated group) immature cartilage formation adjacent to the perichondrial graft was noted. At 4 and 6 weeks, in groups 2 and 3, newly developed immature chondrocyte groups were observed. In these groups, the chondrocytes mostly appeared single, smaller and more pyknotic with no visible capsules. In group 1, mature cartilage formation was noted. The chondrocytes were spherical in shape with vacuoles in the cytoplasm (Fig. 1). At 8 weeks, in all groups, the chondrocytes were mature, however, the cartilage plate in groups 2 and 3 were significantly less thin and extensive than in group 1 (Fig. 2).

The HAF-treated group produced a mean cartilage thickness of 919 μm, saline-treated group produced a mean of 651 μm, and control group produced a mean of 643 μm at 8 weeks (Table 1). The newly formed cartilage plate in the HAF-treated group was significantly thick when compared with groups 2 and 3 ($p < 0.05$, ANOVA). Additionally, no significant difference was found between the groups 2 and 3.

Figure 1 Cartilage formation 4 weeks after the transplantation of the perichondrial graft over the paravertebral muscles of the rabbit. In the saline-treated group (A) and the control group (B), the neocartilage (nc) has mostly immature appearance with single, small and pyknotic chondrocytes with no visible capsules. In the HAF-treated group (C), the neocartilage (nc) has a mature appearance with large lacunae containing spherical chondrocytes surrounded by well-defined capsules (H&E, × 100).



Discussion

Cartilage replacement is important, especially in maxillofacial, orthopaedic and reconstructive plastic surgery. Since several experimental and clinical studies have demonstrated advantages of perichondrial grafting in the treatment of cartilage defects,[1-13] it is of particular interest to examine factors which can promote cartilaginous differentiation from perichondrium. Cell-culture studies demonstrated that growth factors such as IGF, bFGF and EGF have promoting effect on chondrocyte differentiation and proliferation.[25-29] On the other hand, in vitro studies have shown that intact fibronectin, being an extracellular matrix precursor, may contribute to the repair of the matrix of the cartilage by stimulating proteoglycan synthesis.[25] HAF is known to be rich in these growth factors and extracellular matrix precursors during the second trimester of gestation.[25-29,31] In addition to these factors, HAF contains high concentrations of high molecular weight HA and HASA which stimulate the neochondrogenesis from perichondrium.[14-20] These factors contained in HAF may explain the observed acceleration of cartilage regeneration from the perichondrial grafts in the experimental group.

Investigations have shown that no viruses, bacteria, mycoplasma, fungi or chlamydia can be isolated from HAF at the second trimester.[32] It is also known that mean IgG level of HAF is highest during this period. IgG might prevent wound infection and indirectly the formation of scar tissue which is responsible for minimising the extent of cartilage repair.

Since in our previous study it was shown that single dose injection of 0.2 ml HAF was sufficient to enhance the neochondrogenesis,[30] in this study 0.3 ml HAF was injected in order to be sure of the effective quantity of substance and to fill the undersurface of the perichondrial graft adequately. However, it seems that restoration of circulation to the area would quickly remove and dilute the effectiveness of the substance. This is a criticism of all topical application of fluid to the wound site but to apply the solution repeatedly to the surgical area risked infection and it is unlikely that this

Figure 2 Cartilage formation 8 weeks after the transplantation of the perichondrial graft over the paravertebral muscles of the rabbit. The neocartilage plate (nc) in the saline-treated group (A) and control group (B) were significantly thin and less extensive compared with the HAF-treated group (C). (Masson Trichome, × 100).

Table 1 Mean thickness of neocartilage at 8 weeks

Animal No.	Group 1 (μm)	Group 2 (μm)	Group 3 (μm)
1	910	750	800
2	900	830	780
3	850	600	620
4	880	610	640
5	970	500	485
6	950	610	580
7	910	590	630
8	1000	700	520
9	850	750	740
10	960	640	700
11	920	690	580
12	930	540	640
Mean ± SD	919.19 ± 46.21	650.83 ± 95.58	642.91 ± 97.61

would be considered a clinically feasible alternative. Further investigations are needed to find the ideal dose of HAF in cartilage regeneration and to examine possible side effects. Additional studies are also needed to assess the roles of the various factors of HAF in modulating the cartilage-regeneration process.

In conclusion, this experimental study suggests that preoperative injection of HAF underneath the free perichondrial grafts promotes the proliferation and differentiation of the chondrocytes. HAF may promote neochondrogenesis from free perichondrial grafts in the treatment of articular, ear or tracheal cartilage defects.

Acknowledgements

This experimental study was sponsored by the Association of Bursa Plastic Surgery-Burn and Hand Surgery. We would like to express our gratitude to the Clinicians, especially Professor Dr Yalçin Kimya in our Department of Obstetrics and Gynecology at Uludağ University, Bursa, Turkey for providing the human amniotic fluid used in this study.

References

1. Ohlsen L. Cartilage formation from free perichondrial grafts: an experimental study in rabbits. *Br J Plast Surg* 1976;**29**: 262—7.
2. Homminga GN, Bulstra SK, Kuijer R, Linden AJV. Repair of sheep articular cartilage defects with a rabbit costal perichondrial graft. *Acta Orthop Scand* 1991;**62**:415—8.
3. Wasteson A, Ohlsen L. Biosynthesis of chondroitin sulphate in cartilage regenerated from perichondrium. *Scand J Plast Reconstr Surg* 1977;**11**:17—22.
4. Upton J, Sohn SA, Glowacki J. Neocartilage derived from transplanted perichondrium: what is it? *Plast Reconstr Surg* 1981;**68**:166—72.
5. Ohlsen L. Cartilage regeneration from perichondrium: experimental and clinical applications. *Br J Plast Surg* 1978;**62**:507—13.
6. Sohn SA, Ohlsen L. Growth of cartilage from a free perichondrial graft placed across a defect in a rabbit's trachea. *Plast Reconstr Surg* 1974;**53**:55—60.
7. Ruuskanen MM. Shaped regeneration of rabbit ear perichondrium. *Scand J Plast Reconstr Hand Surg* 1991;**25**:193—7.
8. Hartig GK, Esclamado RM, Telian SA. Comparison of the chondrogenic potential of free and vascularized perichondrium in the airway. *Ann Otorhinolaryngol* 1994;**103**:9—15.
9. Brent B, Ott R. Perichondro-cutaneous graft. *Plast Reconstr Surg* 1978;**62**:1—14.
10. Engkvist O, Johansson SH, Ohlsen L, Skoog T. Reconstruction of articular cartilage using autologous perichondrial grafts. A preliminary report. *Scand J Plast Reconstr Surg* 1975;**9**: 203—6.
11. Skoog T, Johansson SH. The formation of articular cartilage from free perichondrial grafts. *Plast Reconstr Surg* 1976;**57**: 1—6.
12. Gültan SM, Emiroglou M, Cenetoglou IS, Yormuk E. Effects of free perichondrial graft replacement of epiphyseal cartilage on bone growth. *Scand J Plast Reconstr Surg Hand Surg* 1993; **27**:1—8.
13. Tajima S, Aoyagi E, Maruyama Y. Free perichondrial grafting in the treatment of temporomandibular joint ankylosis. Preliminary report. *Plast Reconstr Surg* 1978;**61**:876—80.
14. Kawasaki K, Ochi M, Uchio Y, et al. Hyaluronic acid enhances proliferation and chondroitin synthesis in cultured chondrocytes embedded in collagen gels. *J Cell Physiol* 1999;**179**: 142—8.
15. Sonoda M, Harwood FL, Wada Y, et al. The effects of hyaluronan on the meniscus and on the articular cartilage after partial meniscectomy. *Am J Sports Med* 1997;**25**: 755—62.
16. Armstrong S, Read R, Ghosh P. The effects of intraarticular hyaluronan on cartilage and subchondral bone changes in an ovine model of early osteoarthritis. *J Rheumatol* 1994;**21**: 680—8.
17. Bertolami CN, Gay T, Clark GT, et al. Use of sodium hyaluronate in treating temporomandibular joint disorder: a randomized, double-blind, placebo-controlled clinical trial. *J Oral Maxillofac Surg* 1993;**51**:232—42.
18. Abatangelo G, Botti P, Del Bue M, et al. Intraarticular sodium

hyaluronate injections in the Pond-Nuki experimental model of osteoarthritis in dogs. I. Biochemical results. *Clin Orthop* 1989;**241**:278—85.
19. Schiavinato A, Lini E, Guidolin D, et al. Intraarticular sodium hyaluronate injections in the Pond-Nuki experimental model of osteoarthritis in dogs. II. Morphologic findings. *Clin Orthop* 1989;**241**:286—99.
20. Burd DAR, Greco RM, Regauer S, Longaker MT, Siebert JW, Garg HG. Hyaluronan and wound healing: a new perspective. *Br J Plast Surg* 1991;**44**:579—84.
21. Dahl LB, Kimpton WG, Cahill RNP, Brown TJ, Fraser JRE. The origin and fate of hyaluronan in amniotic fluid. *J Dev Physiol* 1989;**12**:209—18.
22. Longaker MT, Adzick S, Hall JL, et al. Studies in fetal wound healing. VII. Fetal wound healing may be modulated by hyaluronic acid stimulating activity in amniotic fluid. *J Pediatr Surg* 1990;**25**:430—3.
23. Çek DI, Ozer K, Perk C, Akalin G, Ozcan G. Histologic changes in capsule formation around silicone implants after a single dose injection of amniotic fluid. *Eur J Plast Surg* 1992;**15**: 289—91.
24. Longaker MT, Ernest SC, Michael RH, et al. Studies in fetal wound healing. IV. Hyaluronic acid-stimulating activity distinguishes fetal wound fluid from adult wound fluid. *Ann Surg* 1989;**210**:669—72.
25. Silver FH, Glasgold AI. Cartilage wound healing: an overview. *Otolaryngol Clin North Am* 1995;**28**:847—64.
26. Merimee TJ, Grant M, Tyson JE. Insulin-like growth factors in amniotic fluid. *J Clin Endocrinol Metab* 1984;**752**:55.
27. Fujisato T, Sajiki T, Liu Q, Ikada Y. Effect of basic fibroblast growth factor on cartilage regeneration in chondrocyte-seeded collagen sponge scaffold. *Biomaterials* 1996;**17**: 155—62.
28. Fujimoto E, Ochi M, Kato Y, Mochizuki Y, Sumen Y, Ikuta Y. Beneficial effect of basic fibroblast growth factor on the repair of full-thickness defects in rabbit articular cartilage. *Arch Orthop Trauma Surg* 1999;**119**:139—45.
29. Shida J, Jingushi S, Izumi T, Iwaki A, Sugioka Y. Basic fibroblast growth factor stimulates articular cartilage enlargement in young rats in vivo. *J Orthop Res* 1996;**14**:265—72.
30. Özgenel GY. The influence of human amniotic fluid on the potential of rabbit ear perichondrial flaps to form cartilage tissue. *Br J Plast Surg* 2002;**55**:246—50.
31. Siebert JW, Burd AR, McCarthy JG, Weinzweig J, Ehrichl HP. Fetal wound healing: a biochemical study of scarless healing. *Plast Reconstr Surg* 1990;**85**:495—502.
32. Miglioli PA, Schoffel U, Gianfranceschi L. The in vitro synergistic inhibitory effect of human amniotic fluid and gentamicin on growth of *Escherichia coli*. *Chemotherapy* 1996;**42**:206—9.