Genotoxicity and cytotoxicity of ovine collagen on human dermal fibroblasts

Fauzi M. Busra, BSc,
Shiplu R. Chowdhury, ME, PhD,
Aminuddin B. Saim, MD, MS,
Ruszymah B. Idrus, MD, PhD.

Scaffold is one of the 3 main components in the field of tissue engineering and regenerative medicine. Fabrication technology is widely used to produce temporary scaffold from biomaterials, either synthetic or natural. Application of implantation technique preferably demands natural biomaterial due to their excellent biocompatibility and biodegradability, as well as low antigenicity. Besides, due to their biomechanical properties, natural biomaterials play a vital role in tissue engineering to provide the suitable strength, modulus, and resistance.[1] This study aimed to extract collagen from ovine tendon, and evaluate its genotoxic and cytotoxic effects on human dermal fibroblast cells (HDFCs) considering the use in skin tissue engineering.

All experimentations in this study were performed from June 2009 to April 2010. This study has been approved by the Ethical Committee of Universiti Kebangsaan Malaysia. Skin samples were obtained from 6 consenting patients as redundant tissue after surgery. The HDFCs were isolated and suspended in equivalent mixture of Ham's F-12: high glucose Dulbecco's Modified Eagle's Medium (DMEM) (FD; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). The cells were incubated at 37^{0}C in humidified atmospheric condition of 5% CO_2 with medium changed every 2-3 days until confluence. The cells were then trypsinized and seeded into 96-well plate for cytotoxic evaluation. The extraction of collagen type I was carried out using the method established by Rajan et al[2] in 2006 with some modifications. In brief, dried tendon from ovine was dissolved in 0.02 M acetic acid at 4^{0}C for 24-48 hours, and centrifuged at 10,000 rpm for 45 minutes. Supernatant containing collagen was collected and neutralized to physiological pH (pH 7) by 1M sodium hydroxide (NaOH) solution, and kept at -30^{0}C for 6-12 hours. This freezed collagen solution was then freeze-dried for 24-48 hours to acquire collagen sponge. The purity of the isolated collagen was not tested in this study. The cytotoxic and genotoxic properties of collagen on the HDFCs was evaluated using indirect method by exposing collagen leachate that was established as a gold standard (ISO 10993; Part 12). To achieve collagen leachate, collagen sponge was dissolved in the culture media (FD + 10% FBS) for 72 hours. Genotoxicity was evaluated by alkaline Comet assay, and the DNA damage was analyzed by comet score software as described by Ribeiro et al 2006.[3] The cytotoxicity of collagen on HDFCs was evaluated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) assay.

Data were expressed as mean ± standard error mean. The results were analyzed using ANOVA for statistical significance with confidence interval of 95% (that is, $p<0.05$ was considered significant).

The cytotoxic effect of collagen on HDFCs was evaluated at various concentrations of 0.15, 0.3, 0.6, 1.3, 2.5, and 5.0 mg/ml of collagen at day 3. The viability decreased with increasing collagen concentration, and at 2.5 mg/ml of collagen the value was 88.2%, and at 5.0 mg/ml of collagen the values was 67.8%. According to standard procedure the viability of more than 50% is considered non-cytotoxic. Taken this into account and considering the production of collagen sponge with higher mechanical strength, the kinetics of cellular growth properties were evaluated at the collagen concentration of 2.5 and 5.0 mg/ml by analyzing viable cells at day 3, 5 and 7 along with control culture (FD without collagen lechate). At 2.5 mg/ml of collagen, the number of viable HDFCs increased gradually with time, and at day 7 it was 100.5%, which was 1.16 times higher than that at day 3 and significantly different ($p<0.05$). The viability at 2.5 mg/ml of collagen were significantly lower compared to the control culture at day 3 and 5. In case of 5.0 mg/ml of collagen concentration, the number of viable cells at day 3 was significantly lower compared to that in the case of 2.5 mg/ml and control culture. However, the number of viable cells was found to increase dramatically at day 5, and decreased at day 7. This data suggested that the isolated collagen from sheep tendon has the concentration-dependent effect on the proliferation of HDFCs in vitro. For evaluation of genotoxic effect of collagen on HDFCs, the concentration of collagen of 2.5 and 5.0 mg/ml were chosen. **Figure 1a** shows the typical morphology of HDFCs nucleus under various treatment conditions. The index for DNA damage was evaluated using comet score software and the index is classified as no DNA damage (class 0), little DNA damage (class 1), medium DNA damage (class 2), damage (class 3), and maximum damage (class 4).[4]

According to the photo micrograph observation, the DNA damage of HDFCs at collagen concentration of 2.5 and 5.0 mg/ml was classified as class 0, indicating no DNA damage. Moreover, percentage of DNA in tail was measured for various treatment conditions (**Figure 1b**). In the case of 2.5 and 5.0 mg/ml of collagen, the score for DNA percentage in tail was found almost similar as control group (<5), those are significantly

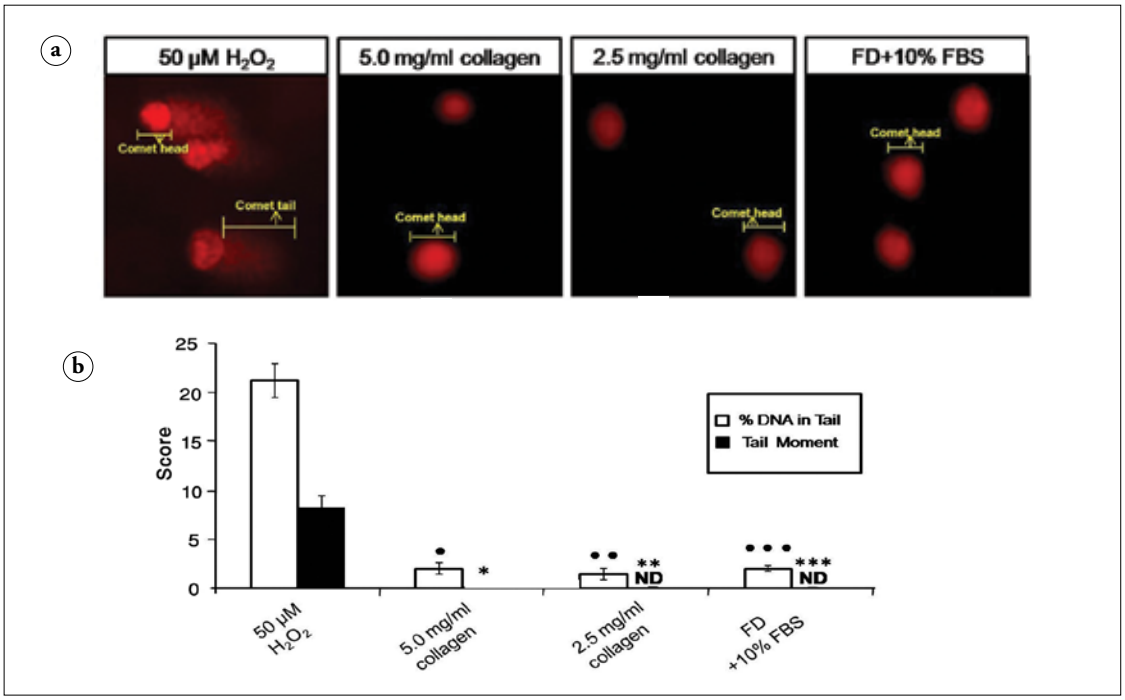

Figure 1 - Genotoxicity effect of sheep collagen on human dermal fibroblast cells (HDFCs): **a**) representative photomicrograph of comet assay for the test conditions (5.0 & 2.5 mg/ml collagen leachate) along with the positive control (50 µM H_2O_2) and contrast condition (FD+10% fetal bovine serum [FBS]), **b**) quantitative evaluation of genotoxic effect by means of percent of DNA in tail (open bar) and tail moment (close bar) for the condition mentioned above. The data were obtained for 6 different HDFCs sample. The black dot (•) and asterisk (*) indicate statistical significance between the respective data sets, which were analyzed by ANOVA with confidence interval of 95%, that is, $p<0.05$. ND - not detected.

lower than the positive control group (>20). According to previous study, the score below 5 is considered to be non-genotoxic to cells. Another genotoxicity evaluation index, the tail moment was also evaluated for various treatment conditions as shown in **Figure 1b**. There are different approaches to analysis of comets, including the scoring classification by arbitrary units.[5] In this study, Lebaily's methods was used and according to this method, the mean tail moment score of less than 1 indicate no damage, 1-5 small damage, 6-60 medium damage, and >60 maximum damage. In this study, the mean tail moment in the case of 2.5 and 5.0 mg/ml of collagen was found almost similar, while significantly higher in case of positive control (>80). This results indicated that collagen at both concentration has no genotoxic effect for HDFCs.

As conclusion, porous sheep collagen sponge was successfully fabricated by employing the freeze-drying technique. There was no cytotoxic and genotoxic effect towards human dermal fibroblast cell even at the highest concentration. Therefore, the collagen extracted from sheep tendon is biocompatible on HDFCs and can be use as a biomaterial in skin tissue engineering.

Acknowledgment. *The authors gratefully acknowledge Associate Prof. Dr. Nor Fadilah Bt. Rajab from the Biomedical Department, Faculty of Health Science, Universiti Kebangsaan, Malaysia for her invaluable advice on the comet assay analysis.*

Received 20th June 2011. Accepted 3rd October 2011.

From the Tissue Engineering Centre (Busra, Chowdhury, Saim, Idrus), UKM Medical Centre, Jalan Yaacob Latiff, Bandar Tun Razak, Cheras, and the Department of Physiology (Idrus), Medical Faculty UKM, Jalan Raja Muda Abdul Aziz, Kuala Lumpur, and the Ear, Nose, and Throat Consultant Clinic (Saim), Ampang Puteri Specialist Hospital, Taman Dato Ahmad Razali, Ampang, Selangor, Malaysia. Address correspondence and reprints request to: Professor Dr. Ruszymah B. Idrus, Head, Tissue Engineering Centre, Universiti Kebangsaan Malaysia Medical Centre, Jalan Yaacob Latif, Bandar Tun Razak, 56000 Kuala Lumpur, Malaysia. Tel. +60 (3) 91457670 Fax. +60 (3) 91457678. E-mail: ruszyidrus@gmail.com/ruszy@medic.ukm.my

References

1. Muschler GF, Nakamoto C, Griffith LG. Engineering principles of clinical cell-based tissue engineering. ***J Bone Joint Surg Am*** 2004; 86: 1541-1558.
2. Rajan N, Habermehl J, Coté MF, Doillon CJ, Mantovani D. Preparation of ready-to-use, storable and reconstituted type I collagen from rat tail tendon for tissue engineering applications. ***Nat Protoc*** 2006; 1: 2753-2758.
3. Ribeiro DA, Matsumoto MA, Duarte MA, Marques ME, Salvadori DM. Ex vivo biocompatibility tests of regular and white forms of mineral trioxide aggregate. ***Int Endod J*** 2006; 39: 26-30.
4. Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. ***Mol Biotechnol*** 2004; 26: 249-261.
5. Collins AR, Oscoz AA, Brunborg G, Gaivao I, Giovannelli L, Kruszewski M, Smith CC, Stetina R. The comet assay: topical issues (Review). ***Mutagenesis*** 2008; 23: 143-151.