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Decellularized and Genipin Crosslinked Human Umbilical Cord Artery and Vein for Potential Use as Peripheral Nerve Conduit

(Pautan Silang Arteri dan Urat Tali Pusat Manusia Dinyahsel dan Genipin untuk Potensi Kegunaan sebagai Konduit Saraf Periferi)

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ABSTRACT

Critical gap peripheral nerve injury, commonly caused by motor vehicle accidents, results in dysfunctional nerve and impaired body function. Our study aims to develop a conduit from decellularized and genipin crosslinked human umbilical cord artery and vein for future use in critical nerve gap injury treatments. Human umbilical cord arteries (HUCA) and veins (HUCV) were divided into native (nHUCA and nHUCV), decellularized (dHUCA and dHUCV) and genipin-crosslinked (clHUCA and clHUCV) groups. Both the decellularized and crosslinked groups were decellularized, and subsequently, the clHUCA and clHUCV groups were crosslinked with 0.1%, 0.4% and 0.7% (w/v) genipin. The HUCA and HUCV were then studied for decellularization efficiency, crosslinking index, biodegradation, swelling ratio, ultrastructure analysis, flexibility and mechanical strength. In addition, mesenchymal stem cells isolated from Wharton's jelly were seeded into HUCA and HUCV for biocompatibility studies. The degradation test showed that nHUCV and dHUCV degraded at day 7 compared to other groups that did not show any degradation even after 21 days. Biocompatibility studies showed that the conduits crosslinked with 0.4% (w/v) genipin were successfully seeded and was having the most amount of seeded cells. In conclusion, the decellularization and genipin crosslinking of human umbilical cord artery and vein enabled successful in fabrication of conduit with suitable properties such as reduced swelling, flexibility, porosity and mechanical strength, with potential in tissue engineering applications.

Keywords: Decellularization; genipin; nerve conduit; nerve injury; umbilical cord artery

ABSTRAK

Kecederaan saraf periferi jurang kritikal, biasanya disebabkan oleh kemalangan kenderaan bermotor mengakibatkan saraf tidak berfungsi dan fungsi badan akan terjejas. Kajian ini bertujuan untuk membangunkan konduit daripada arteri dan urat tali pusat manusia yang dinyahsel dan genipin untuk kegunaan masa hadapan dalam rawatan kecederaan jurang saraf kritikal. Arteri tali pusat manusia (HUCA) dan urat (HUCV) dibahagikan kepada kumpulan asli (nHUCA dan nHUCV), dinyahsel (dHUCA dan dHUCV) dan pautan silang genipin (clHUCA dan clHUCV). Kedua-dua kumpulan dinyahsel dan pautan silang telah dinyahsel dan seterusnya, kumpulan clHUCA dan clHUCV telah dipaut silang dengan 0.1%, 0.4% dan 0.7% (w/v) genipin. HUCA dan HUCV kemudiannya dikaji untuk kecekapan dinyahsel, indeks paut silang, biodegradasi, nisbah bengkak, analisis ultrastruktur, kefleksibelan dan kekuatan mekanikal. Di samping itu, sel stem mesinkima yang diasingkan daripada jeli Wharton telah disemai ke dalam HUCA dan HUCV untuk kajian biokeserasian. Ujian degradasi menunjukkan nHUCV dan dHUCV merosot pada hari ke-7 berbanding kumpulan lain yang tidak menunjukkan sebarang degradasi walaupun selepas 21 hari. Kajian biokeserasian menunjukkan bahawa konduit yang dipaut silang dengan genipin 0.4% (w/v) berjaya dibenih dan mempunyai jumlah sel yang paling banyak. Kesimpulannya, dinyahsel dan pautan silang genipin arteri dan urat tali pusat manusia telah berjaya menghasilkan konduit dengan sifat yang sesuai seperti mengurangkan bengkak, kefleksibelan, keliangan dan kekuatan mekanikal dengan potensi dalam aplikasi kejuruteraan tisu.

Kata kunci: Arteri tali pusat; dinyahsel; genipin; kecederaan saraf; konduit saraf

Introduction

Peripheral nerve injury is a significant critical issues in hospitals that may lead to permanent disability, which could give an effect on a patient's quality of life (Wojtkiewicz et al. 2015). There are several causes of peripheral nerve injury, such as direct trauma on the nerve due to works-site injuries, road accidents, gunshot wounds, falls, sports injuries, tumours and iatrogenic procedures (Wood et al. 2011).

Various studies had utilized biological conduits to treat peripheral nerve injury (Hussin, Idrus & Lokanathan 2018), especially in critical nerve gaps that are greater than 3 cm in humans. Use of autologous nerve graft have been the gold standard to bridge the gaps that are irreparable by direct suturing alone (Lokanathan et al. 2014). Nevertheless, the autologous nerve graft is not readily obtainable as the surgery to obtain the autologous sural nerve graft could result in harvest-site scarring, morbidity, sensory, and functional loss of the donor nerve site and neuroma formation. Other limitations include limited availability and size mismatch of donor graft (Gordon, Sulaiman & Boyd 2003; Zhan et al. 2013). The drawbacks of nerve autograft have necessitated study for substitutes in bridging nerve gaps, such as nerve conduits of natural and synthetic origin. Synthetics nerve conduits have several disadvantages such as causing persistent inflammation, acidic breakdown by-product and stiffness mismatch (Deal, Griffin & Hogan 2012; Liao et al. 2013). As a result, researchers have investigated the use of nerve conduits from natural biological sources (Geuna et al. 2014; Hussin, Idrus & Lokanathan 2018).

In nerve bridging using conduit, proximal and distal parts of the severed nerve would be inserted into the opposite ends of a nerve conduit to guide the axons to regenerate from the proximal ends towards the distal end while being entubulated inside the nerve conduit (Muheremu & Ao 2015). In addition, the improved conduits may also supplement neurotrophic factors and provide microenvironment that promote nerve regeneration while avoiding ingrowth and fibrosis of surrounding tissue (Muheremu & Ao 2015).

This study aimed to develop conduit from decellularized and genipin crosslinked human umbilical cord artery and vein, suitable for use as peripheral nerve conduits. The development of peripheral nerve conduits from human umbilical cord artery and vein was optimized from a previous study (Hussin, Idrus & Lokanathan 2018) by crosslinking the conduits with genipin, and were further characterized *in vitro*. Although the ECM is retained and some antigens might be present in it, previous studies found that these scaffolds do not induce immunogenic reaction during allogenic or xenogeneic grafting (Mirmalek-Sani et al. 2013). Furthermore, as we are using human tissues, the completely decellularized tissues have a very low immunogenicity as the major histocompatibility complex (MHC) antigens that are present on the surface of cells would have been removed during the decellularization process (Massaro et al. 2021).

Genipin shown to be superior crosslinking agent because it is more biocompatible and less cytotoxic compared to other chemical crosslinkers, and produce superior mechanical strength and degradation index (Yoo et al. 2011; Yuan et al. 2007). In this study, the decellularized human umbilical cord artery and vein was crosslinked with genipin and its characteristics, namely the (1) decellularization efficiency, (2) crosslinking index, (3) biodegradation rate, (4) swelling rate (5) ultrastructure and pore size of the conduit, (6) biocompatibility, (7) flexibility, as well as (8) mechanical strength were studied.

Materials and Methods

Human umblical cord collection and processing

Ethics approval was obtained from Universiti Kebangsaan Malaysia Research Ethics Committee (UKMREC) (UKM PPI/111/8/JEP-2020-208). The human umbilical cord (HUC) samples (n = 6) were obtained from healthy donors undergoing full-term elective caesarean delivery. The inclusion criteria were normal morphology of the cord from full-term elective caesarean delivery with no evident complication of the newborn or birth. This was to ensure uniform samples and to provide samples with minimal contamination.

Decellularization of human umbilical cord artery and vein

After collecting the sample, the HUC was rinsed multiple times with sterile Dulbecco's phosphate buffer saline (DPBS) (Sigma Aldrich, USA) to remove any blood stains. The HUC samples' arteries and veins were dissected and pulled away. The Wharton's jelly portion was preserved to isolate mesenchymal stem cells. The HUC arteries and veins were decellularized according to a previously reported methodology (Sun et al. 2011). The arteries and veins were cleansed in sterile ice-cold saline after being cleaned of blood and connective tissue. The cells were then decellularized using a variety of enzymes and detergents, including 1% detergent Triton X-100 (Sigma Aldrich, USA) and 1% sodium dodecyl sulphate (SDS) (Sigma Aldrich, USA) based on a prior work (Hussin, Idrus & Lokanathan 2018).

The Hematoxylin and Eosin (H&E) staining was used following the previously reported protocol (Hussin, Idrus & Lokanathan 2018). After dehydration, tissue sections were permeabilized with 0.5 percent Triton X-100 for 20 minutes and treated with DAPI (1/15000) for 40 minutes in the dark at room temperature to counterstain nuclei with 4′,6-diamidino-2-phenylindole (DAPI) stain. The tissues were then mounted and examined using a fluorescent microscope (Nikon, ECLIPSE Ti) (Hussin, Idrus & Lokanathan 2018).

CROSSLINKING OF DECELLULARIZED SAMPLE BY GENIPIN

The decellularized samples were immersed in 0.1%, 0.4% and 0.7% (w/v) genipin solution for 24 hours at room temperature. Then, the samples were washed with DPBS and stored in DPBS with 2% Antibiotic-Antimycotic (AA) at 80 °C (Gobinathan et al. 2018).

CROSSLINKING INDEX OF DECELLULARIZED SAMPLE BY GENIPIN

The ninhydrin assay was used to determine the amount of free amino groups in each HUC artery and vein. The assay was conducted as described previously (Lai et al. 2012). The optical absorbance of the solution was recorded with an ultraviolet-visible spectrophotometer (Bio-TEK, USA) at 570 nm.

IN VITRO DEGRADATION STUDY

Collagenase Type I (ColI; Worthington, USA) was used to study the degradation rate. The samples were cut into 2 cm in length, and it was incubated in 1 mL of 0.01% ColI, at 37 °C in a humidified 5% CO_2 incubator (New Brunswick Galaxy® 170 Series, Eppendorf, USA). The results were recorded with a digital camera every 7 days from day 0 until day 21 (Gobinathan et al. 2018).

ISOLATION AND PROPAGATION OF HUMAN WHARTON'S JELLY MESENCHYMAL STEM CELLS (hWJMSCs)

The human Wharton's Jelly was isolated from the human umbilical cord. The samples were processed and cells were cultured as described previously (Hussin, Idrus & Lokanathan 2018). Briefly, the collected tissue was minced and digested with 0.6% ColI for 2 h. Then, the digested tissue was centrifuged, washed and cultured in low-glucose Dulbecco's modified Eagle medium (DMEM-LG; Gibco, USA) complete medium (containing 10% fetal bovine serum (FBS; Gibco, USA) and 2% antibiotic-antimycotic (AA; Gibco, USA). Culture mediums were changed every 3 days. Trypsinization was done using TrypLE Select (Gibco, USA). Throughout the experiment, passage 3 cells were used to ensure consistency.

SWELLING ASSAY

The samples were cut into 2 cm in size and were incubated in DMEM-LG complete medium containing 10% FBS and 2% AA at 37 °C in humidified 5% CO2 incubator. The length of the samples was measured every 2 weeks up until week 4 (Hussin, Idrus & Lokanathan 2018).

ULTRASTRUCTURE AND PORE SIZE OF CONDUIT

The samples for arteries and veins from different groups were prepared and the surface morphology and ultrastructure were observed using scanning electron microscopes (SEM). Before observation, the samples were fixed at 4 °C for at least 2 weeks with glutaraldehyde and then washed with DPBS three times. Secondary fixation, serial dehydration and sample mounting with silver paste and sputter coating with gold were done as reported previously (Gobinathan et al. 2018). The gold-coated samples were visualized using Quanta™ 450.

FEG (Scanning Electron Microscope) at Microscopy Imaging Center, Faculty of Pharmacy, Universiti Teknologi MARA, UiTM Puncak Alam. To determine the pore size of each conduit, the mean of ten different pore sizes were calculated.

MECHANICAL STRENGTH TEST

Before mechanical characterization, samples were air-dried and then cut into pieces with a length of 2 cm. Each sample was clamped in between two pieces of sandpaper. At 0.5 mm/min crosshead velocity, the samples were subjected to uniaxial load until they were broken using a 5 kN load cell. The force (in kgf) and elongation (in mm) were recorded using an Instron® 8874 machine (Instron, United Kingdom) to analyse maximum load and extension. The ultimate mechanical strength of each sample was recorded by obtaining the result of maximum load and extension (Gobinathan et al. 2018).

FLEXIBILITY

A kink test was conducted on the conduit by bending the conduit on a flexible 0.6 mm diameter wire until a visible kink or collapse in the lumen. The conduit was bent at a certain degree for 5 minutes until a visible collapse in the lumen diameter occurred. The kink angles were measured by using a protractor (Clements et al. 2016).

BIOCOMPATIBILITY

MSCs from the approximately 0.75 cm cut arteries and veins were seeded into the conduit. Using a p200 micropipette, the cell pellet containing 0.5×10^6 MSCs was resuspended in 20 L of DMEM-LG complete medium comprising 10% FBS and 2% AA

and seeded directly into the lumen of the conduit. To allow cell adhesion, the MSCs seeded conduit (MC) was grown for another two days at 37 °C in a humidified 5% CO_2 incubator. The MC was first fixed with 4% paraformaldehyde (PFA) for 24 hours after 2 days of incubation. To test cell attachment on MSCs planted conduit, H&E staining was conducted as previously reported (Hussin, Idrus & Lokanathan 2018).

Results

DECELLULARIZATION EFFICIENCY

DAPI and H&E staining showed that the nuclei material was absent in decellularized groups compared to native artery and vein as shown in Figure 1.

CROSSLINKING INDEX

The genipin crosslinking was done to improve the mechanical strength and reduce the biodegradation time. Increased genipin concentration from 0.4% to 0.7% was observed to increase the crosslinking degree in both HUCV and HUCA but increased genipin concentration from 0.1% to 0.4% was observed to decrease the crosslinking degree in both HUCV and HUCA, as shown in Figure 2. But the changes in the crosslinking index of HUCA and HUCV across the genipin concentrations tested were statistically insignificant. Thus, a genipin concentration dependent increase of the crosslinking degree was not observed. However, we did not perform neither the flexibility test to check the flexibility or the ability of the conduit to return to its original shape as the kink test give an idea on the flexibility of the conduits.

BIODEGRADATION

On day 0, the human umbilical cord arteries and veins physical appearance were observed and recorded (Figure 3). For the human umbilical cord artery (HUCA), throughout the 21 days, all 5 of the sample groups (nHUCA, dHUCA, 0.1% clHUCA, 0.4% clHUCA and 0.7% clHUCA) does not show any signs of degradation (Figure 3).

For the human umbilical cord vein (HUCV), throughout the 21 days, the native (nHUCV) and the decellularized (dHUCV) groups show obvious degradation (Figure 3). Meanwhile, for all of the crosslinked groups (0.1% clHUCV, 0.4% clHUCV and 0.7% clHUCV), they maintained a physically intact structure even after 21 days (Figure 3).

SWELLING ASSAY

For the human umbilical cord artery (HUCA), throughout 4 weeks there was no increase in size for the native (nHUCA) and all the crosslinked groups (0.1% clHUCA, 0.4% clHUCA and 0.7% clHUCA) (Figure 4). However, there was about 0.5 cm + 0.2 increase in size for the decellularized (dHUCA) group.

For the human umbilical cord vein (HUCV), there was also no increase in size for the native (nHUCV) and all the crosslinked (0.1% clHUCV, 0.4% clHUCV and 0.7% clHUCV) groups even after 4 weeks. However, there was about 0.3 cm + 0.12 increase in size for the decellularized (dHUCV) group.

Figure 4 shows the average and standard deviation for the swelling assay parameters of the human umbilical cord artery (HUCA) and vein (HUCV) (n=6). For HUCA, there was no increase in size for all groups except decellularized HUCA (0.5 cm +- 0.2 increase) throughout 4 weeks. For HUCV, there was also no increase for all groups except decellularized HUCV (0.3 cm +- 0.12 increase) throughout 4 weeks.

ULTRASTRUCTURE & PORE SIZE

The ultrastructure, surface morphology and pore size of the conduit were performed under SEM. However, the underlying cell and extracellular matrix morphology and arrangement could not be observed at most of the sites at 10 000× magnification, as the surface structure of nHUCA and nHUCV was compact (Figure 5).

Besides, dHUCA and dHUCV have smoother surfaces compared to other crosslinked groups. Under 20 000× magnification, the pores on the decellularized and crosslinked conduit had irregular patterns, varied pore sizes and were very interconnected. The pores remained in the crosslinked conduit even after genipin crosslinking was done. The size of the interfibrillary space increased and fibre arrangements are less condensed which leads the conduit to have a larger pore size. The pore size was significantly increase in 0.7% clHUCA compared to nHUCA, 0.1% clHUCA and 0.4% clHUCA ($p<0.05$) (Figure 6). There is also a significant increase in pore size of 0.7% clHUCV compared to the other four groups ($p<0.05$).

BIOCOMPATIBILITY

Biocompatibility studies were conducted by seeding mesenchymal stem cells into the lumen of all 5 groups of the HUCA (Figure 7(a)) and HUCV (Figure 7(b)) which was then incubated for 2 days. The presence of the seeded cells within the lumen and inner wall of the conduit were shown by H&E staining.

FLEXIBILITY

The result showed that the HUCA and HUCV were still flexible up to 65° and 55°, respectively, after decellularization and crosslinking with genipin (Figure 8).

MECHANICAL STRENGTH TEST

The mechanical strength test showed no significant increase in the mechanical strength of the clHUCA and clHUCV groups compared to the native and decellularized group (Figure 9). In addition, the samples in vein groups showed large variation in the mechanical strength.

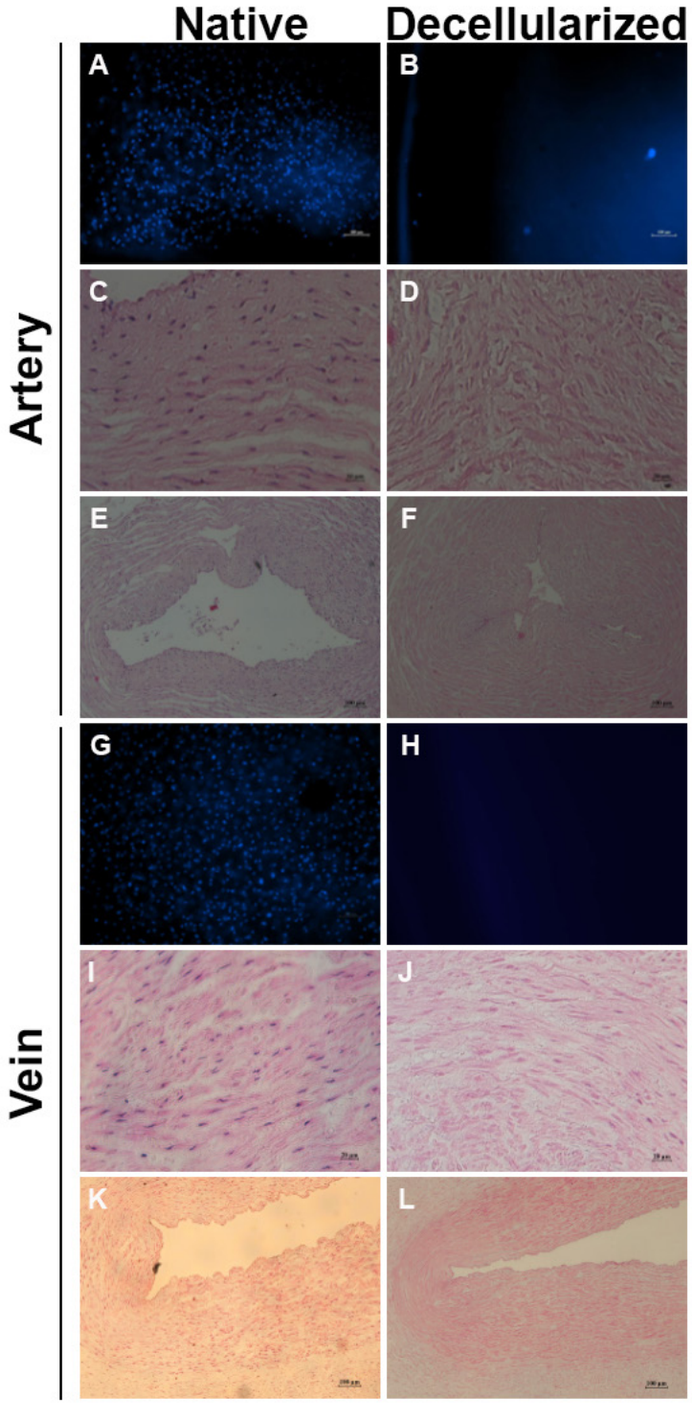

FIGURE 1. DAPI (A, B), H&E 20X magnification (C, D) and H&E 5X magnification (E, F) staining of native and decellularized artery. DAPI (G, H), H&E 20X magnification (I, J) and H&E 5X magnification (K, L) staining of native and decellularized vein. Nuclei counterstaining with DAPI and H&E staining showed that the nuclear materials were removed in decellularized artery (B, D and F) and decellularized vein (H, J and L) compared to native artery (A, C and E) and native vein (G, I and K), respectively. DAPI magnification: 10X, scale bar: 100 µm. H&E magnification: 5X, scale bar: 100 µm; 20X, scale bar: 20 µm

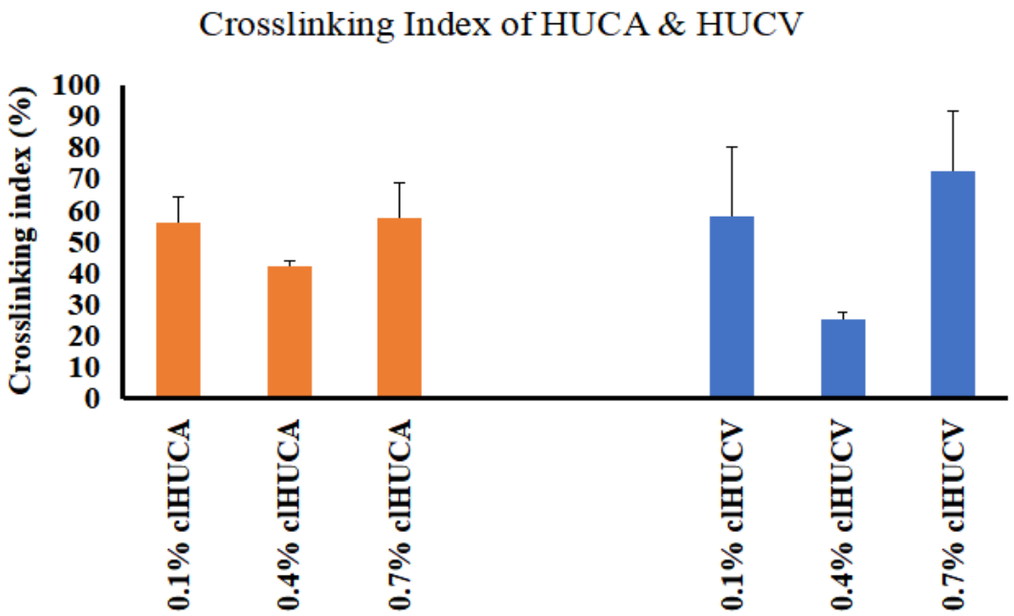

FIGURE 2. The highest crosslinking index was found in 0.7% clHUCA and clHUCV group, whereas the lowest crosslinking index was found in 0.4% clHUCA and clHUCV. No significant difference was observed

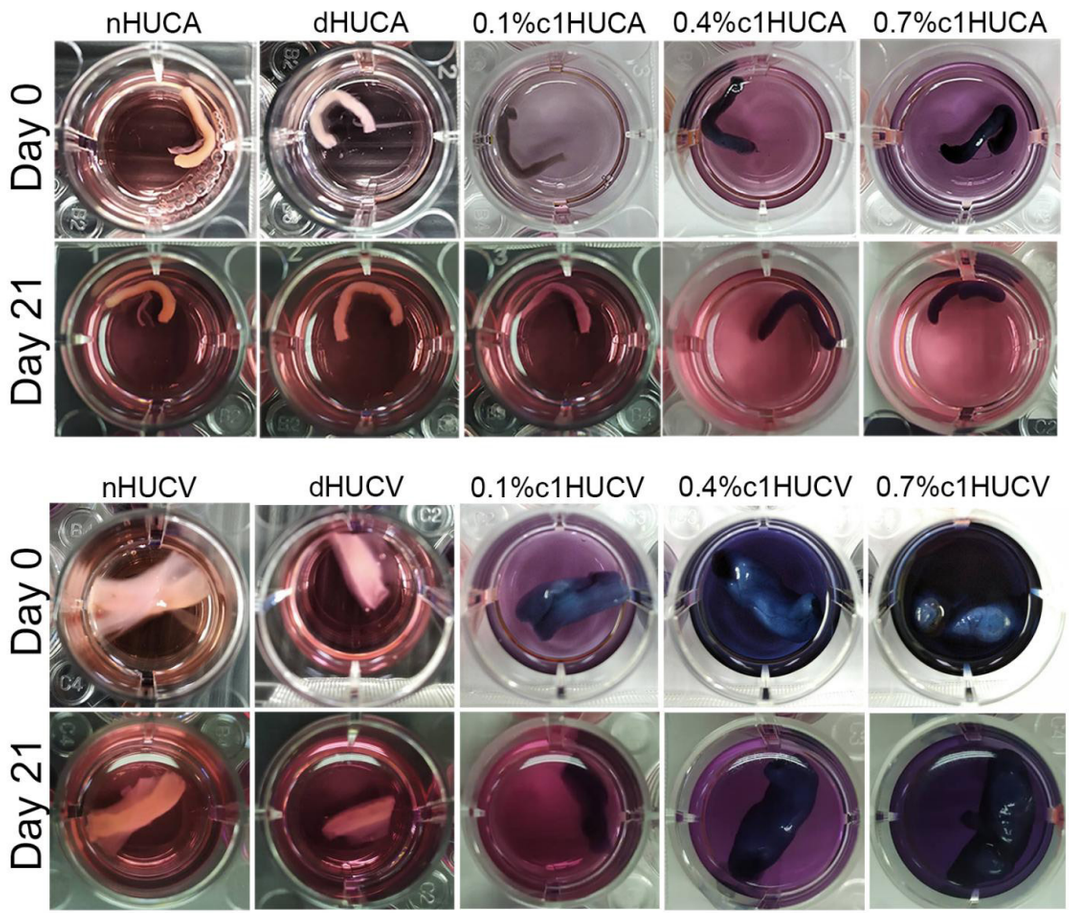

FIGURE 3. *In vitro* degradation assay of the native, decellularized and all the crosslinked human umbilical cord (a) artery (HUCA) and (b) vein (HUCV) in a 0.01% Collagenase Type I enzyme

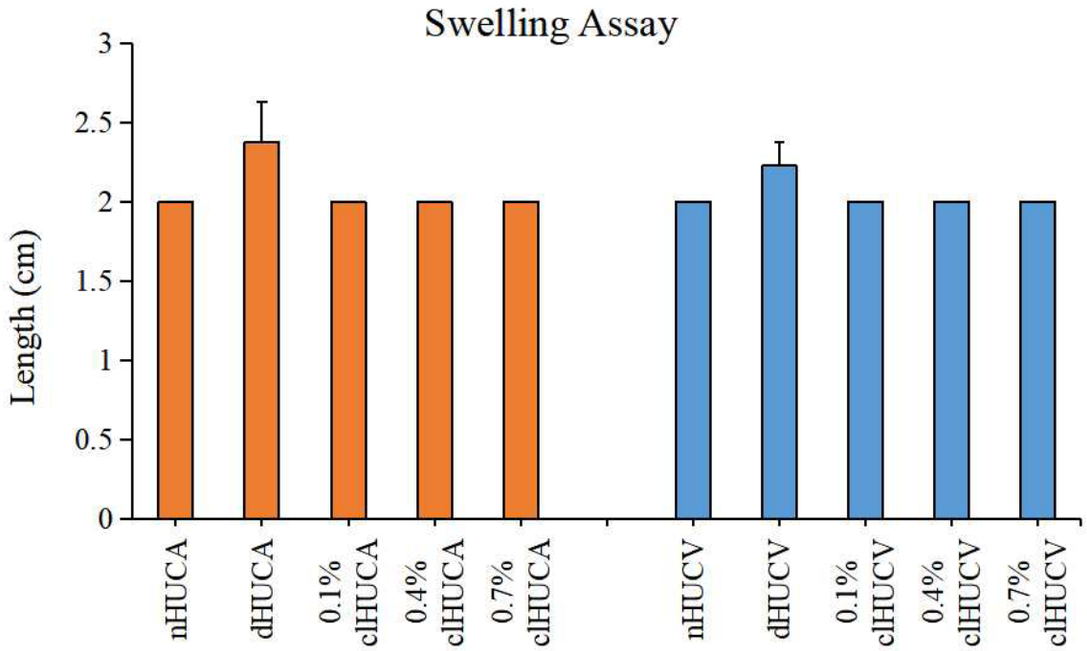

FIGURE 4. The average and standard deviation for the swelling assay parameters of the human umbilical cord artery (HUCA) and vein (HUCV) with (n=6)

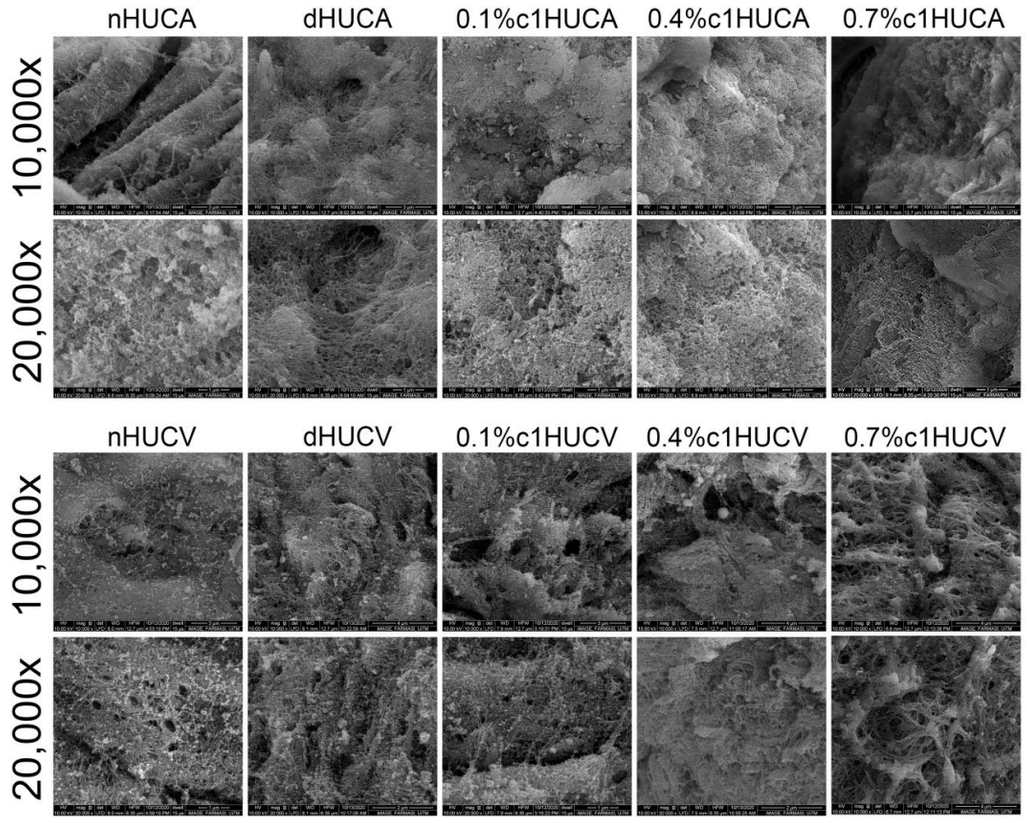

FIGURE 5. Surface ultrastructure analysis of the human umbilical cord artery (HUCA) and vein (HUCV) using SEM under 10,000 and 20,000 magnification

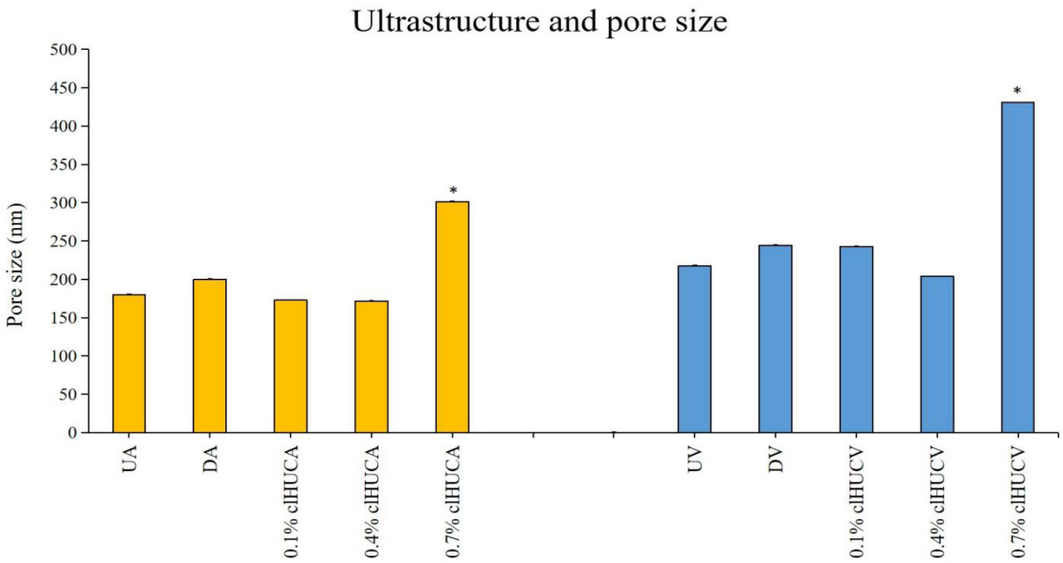

FIGURE 6. The figure shows the average of ten pore sizes of the different groups. The pore size of 0.7% clHUCA was significantly larger compared to other four groups of HUCA ($p<0.05$) and the pore size of 0.7% clHUCV was significantly larger compared to other four groups of HUCV ($p<0.05$)

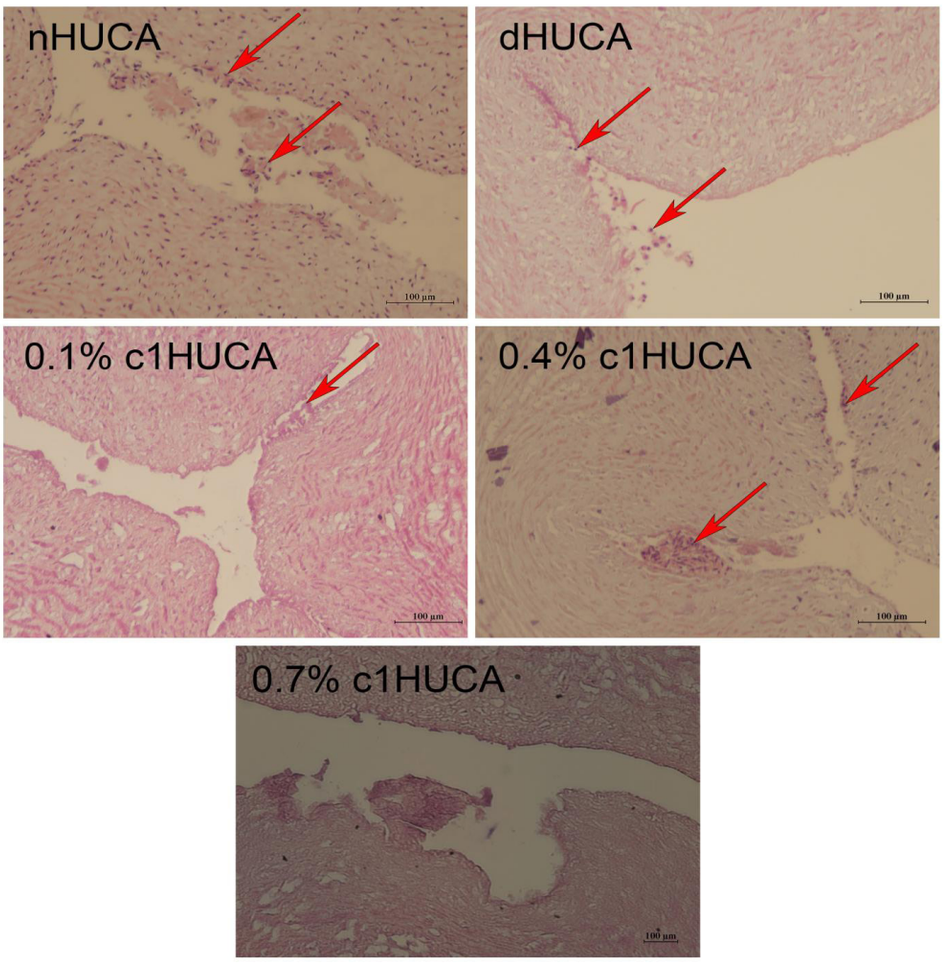

FIGURE 7 (a). H&E staining of the 5 groups of the HUCA samples under light microscope with 10× magnification. The red arrows indicate presence of the seeded cells in the lumen and surface of the conduit. The scale bar=100 µm

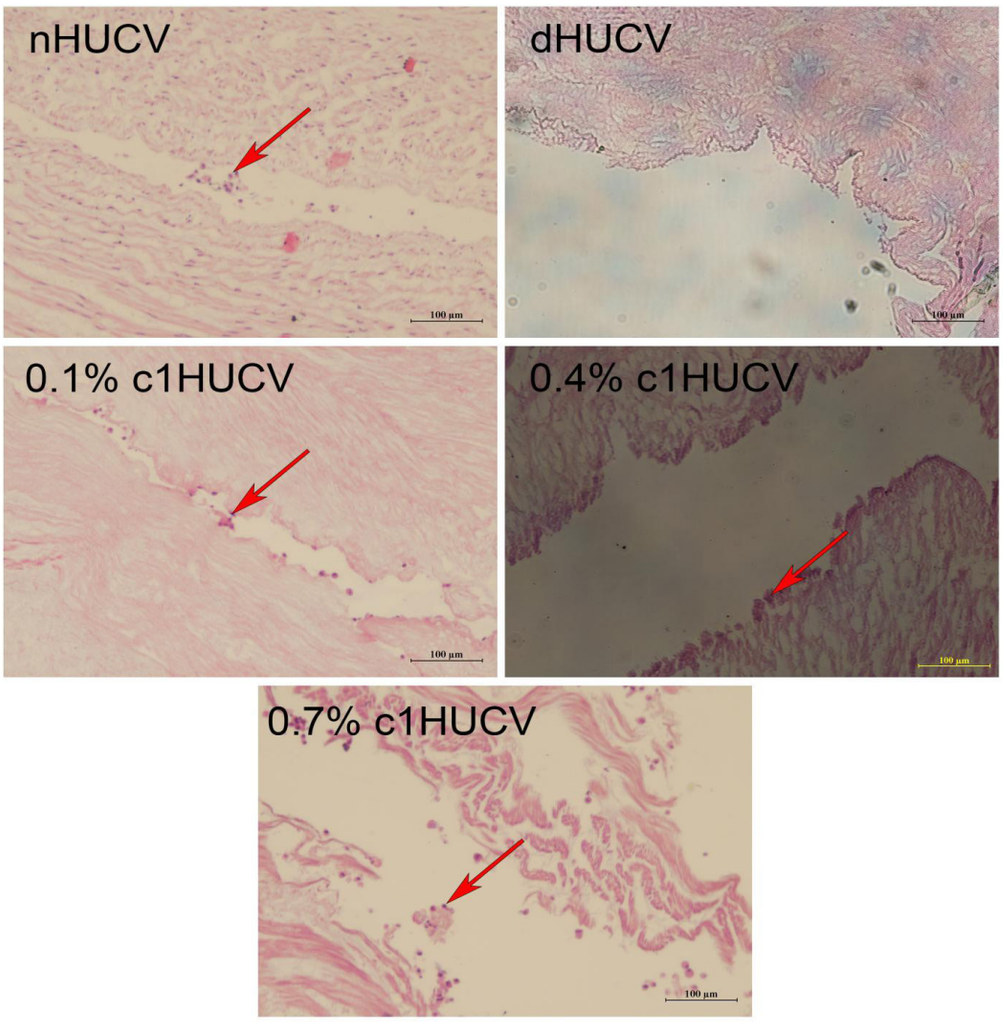

FIGURE 7 (b). H&E staining of the 5 groups of the HUCA samples under light microscope with 10× magnification. The red arrows indicate presence of the seeded cells in the lumen and surface of conduit. The scale bar=100 µm

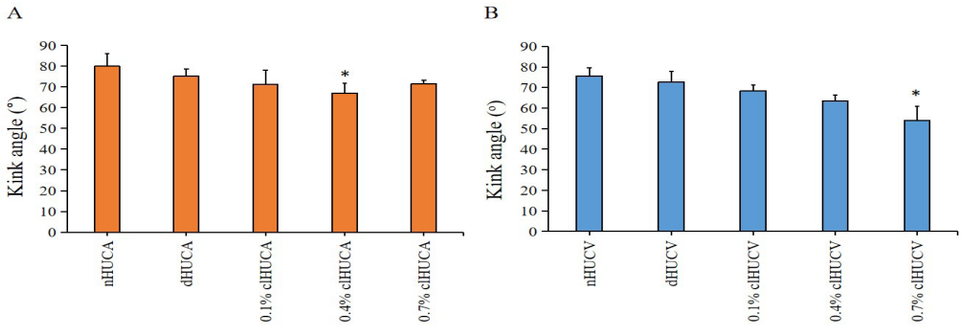

FIGURE 8. (A) Kink angle of various HUCA groups (n = 6). The flexibility was significantly decrease in 0.4% clHUCA compared to native HUCA (p = 0.029). (B) Kink angle of various HUCV groups (n = 6). The flexibility significantly decreased in 0.7% clHUCV compared to the native HUCV (p = 0.0013), decellularized HUCV (p = 0.004) and 0.1% clHUCV (p = 0.0225). * *p*-value<0.05

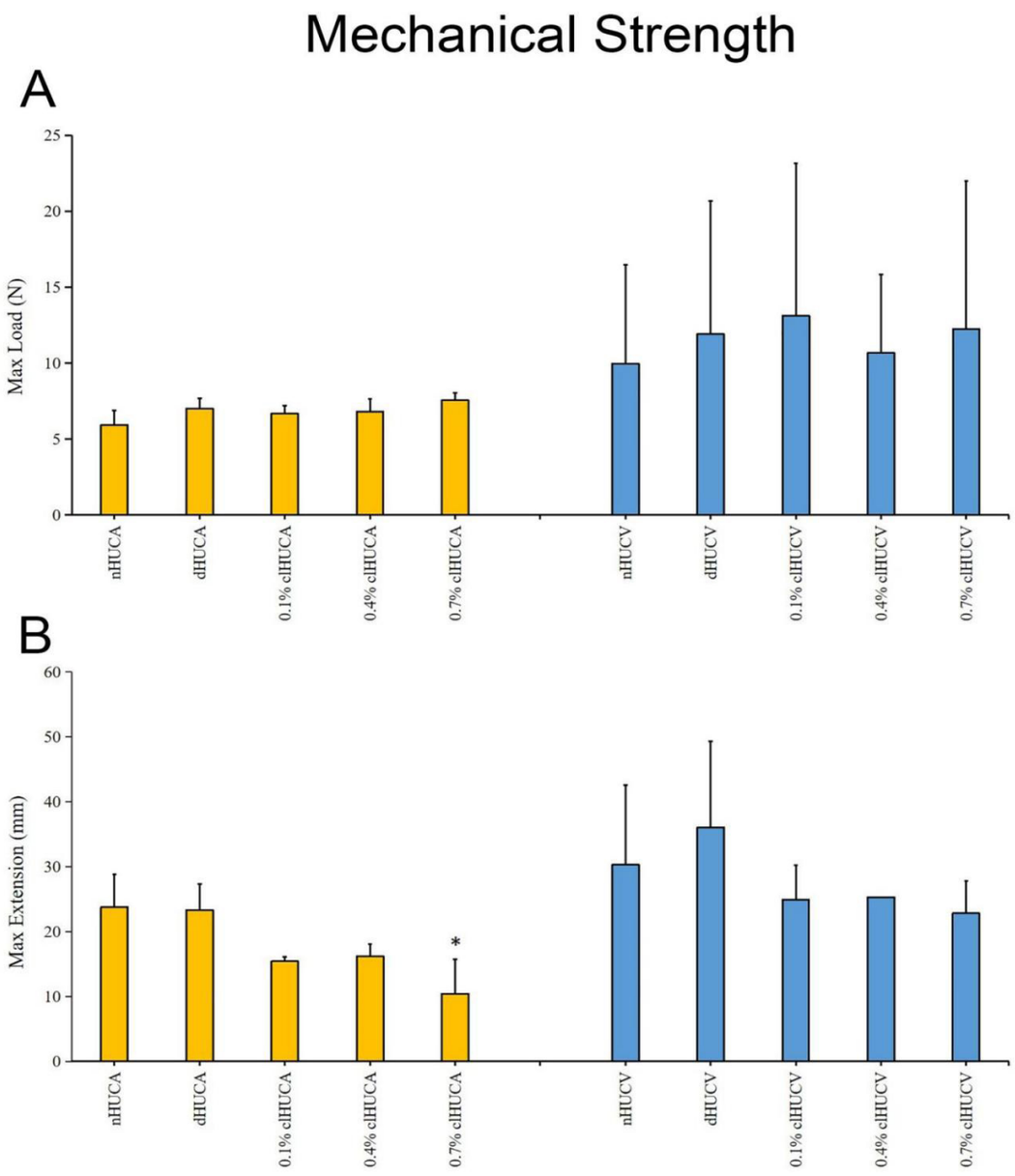

FIGURE 9. Mechanical strength of HUCA measured by the maximum load (A) and extension (B) of the conduit (n=3). There was no significant difference in mechanical strength for maximum load meanwhile for maximum extension there was a significant decrease in 0.7% clHUCA compared to the native HUCA ($p = 0.0118$), decellularized HUCA ($p = 0.0146$)

DISCUSSIONS

In order to construct an ideal nerve conduit, the efficacy of the human umbilical cord decellularization procedure is critical in reducing cellular debris and immunogenicity. This research looked at the formation of nerve conduits utilising decellularized human umbilical cord arteries and veins that were crosslinked with genipin and seeded with hWJMSCs in order to bridge the peripheral nerve gap. H&E staining, nuclei counterstaining with DAPI, conduit length measurement, and *in vitro* evaluation of

constructed conduit were undertaken to investigate the usefulness of a developed nerve conduit.

The aim of the decellularization technique was to eliminate all cellular and nuclear material. As a result, the conduit's decellularization effectiveness was assessed by staining the nuclear and cellular components. The nuclear material was successfully eliminated by the decellularization procedure, as shown by DAPI and H&E staining. According to a recent study, decellularization using a 1 percent (w/v) SDS solution was efficient in lowering cellular content (Ott et al. 2008) and resulted in the removal of immunogenic cells, which was necessary to avoid any subsequent rejection during clinical use (Crouzier et al. 2009). H&E staining showed cytoplasmic, nuclear, and extracellular matrix characteristics.

Genipin, obtained from the fruit of *Gardenia jasminoides*, is a more recently found natural cross-linking agent. It characteristically gives a deep blue colour to primary amino acids, such as the lysine residues of proteins. Hence, when the samples were crosslinked with the genipin, it turns the samples into blue colour (Tomasula 2009). Initially, it was predicted that the higher concentration of genipin, the higher the crosslinking index. However, the result obtained was not as expected. The results showed that 0.4% clHUCA has the lowest crosslinking index instead of 0.1% clHUCA but the difference was not significant. This is probably due to variation in the samples weight that was difficult to control. Ninhydrin that was originally yellow reacted with the free alpha-amino group, NH_2-C-COOH, and turns to deep purple, which is then quantitated by spectrometry (Hwang & Ederer 1975).

The study for biodegradation rate was conducted to examine the biostability of the umbilical cord artery and vein before and after crosslinking with the genipin. At first, it was hypothesized that the genipin crosslinked samples are more resistant to enzymatic degradation compared to the native and decellularized samples. This is true for the human umbilical cord veins where the crosslinked (clHUCV) group was found to be intact even after 21 days, whereas, the native (nHUCV) and decellularized (dHUCV) groups were found to be degrading throughout the 21 days. The degradation of nHUCV and dHUCV were consistent with previous studies where the native and decellularized groups showed biodegradation within 7 days of treatment. However, the decellularized group degrades at a much faster rate. This is due to the disruption and damage caused when they were subjected to a rough 12-hours decellularization process. Meanwhile, the human umbilical cord arteries were intact for all the sample groups even after 21 days. It is concluded that this is due to them having a thick muscular wall as well as the usage of low concentration Collagenase Type I enzyme, which was 0.01%. Genipin successfully increased the biostability of the decellularized HUC in resisting enzymatic degradation. Crosslinking stabilizes the triple-helix structure of collagen, masks collagen cleavage sites and physically hinders the penetration of enzymes into biological tissues (Gobinathan et al. 2018).

A swelling assay was conducted to examine the increase in the size of human umbilical cord arteries and veins after they were incubated in a culture medium for 4 weeks. After 4 weeks, there was only an increase in size for the decellularized groups for both HUCA and HUCV of about 0.5 cm + 0.2 and 0.3 cm + 0.12, respectively. The other HUCA and HUCV groups did not grow in size, and their shape and three-dimensional features remained unchanged. Because the ECM was retained during the decellularization procedure, the length and shape of the decellularized HUC artery conduit remained constant.

A sufficient porosity in conduit helps in cell penetration and adherence, as well as allowing nutrients uptake and waste product elimination from living cells residing inside the scaffold. The pore size of the conduit depends on its fibre thickness. When the fibre thickness decreases, the overall pore size of the conduit increases. It is presumed that the fibres swells upon decellularization process and then shrink upon subsequent crosslinking. This supports the significant increase of fibre thickness after the decellularization process followed by a significant decrease of fibre thickness after genipin crosslinking process. The fibre thickness for the native conduit returned to its original compared to crosslinked groups (Gobinathan et al. 2018). Furthermore, the binding of the crosslinked group fibres was noticeable under the scanning electron microscope (SEM), especially the outermost fibres. This caused crosslinked conduits to be more porous compared to the other groups. Previous studies also have reported that the crosslinked tissues have a more porous surface compared to the decellularized tissues (Hussin, Idrus & Lokanathan 2018).

The seeding of cells into the nerve conduit is critical in supporting nerve regeneration (Kaizawa et al. 2017). Hence an ideal nerve conduit should encourage recellularization. The conduits were placed in 4% paraformaldehyde for 24 hours before H&E staining after 2 days of cell seeding and incubation. The mesenchymal stem cells adhered and spread well in the lumen and

surface of the conduit, according to HUCA and HUCV. The HUCA and HUCV conduits retained the bulk of the extracellular matrix and were able to support the planted cells, demonstrating their biocompatibility. The majority of the conduits had attached cells, although the untreated and 0.4 percent crosslinked groups had the highest number of associated cells, as seen in Figure 6. This also demonstrates that the extracellular matrix collagen fibres, which are important for defining biocompatibility and biomechanical qualities, were retained during the decellularization process, allowing mesenchymal stem cells to adhere to the conduits' walls. Cell adhesion, motility, proliferation, and adaptive tissue responses are all aided by the extracellular matrix (Badylak, Freytes & Gilbert 2009).

MSCs from Wharton's jelly promote Schwann cells regeneration and growth of previous axons, which encourages its use in a nerve conduit. hWJMSCs were chosen as it is easily available and does not have a major ethical concern compared to other MSCs sources, such as bone marrow or peripheral blood. Based on an immunophenotyping study, hWJMSCs also contain unique properties of both human mesenchymal stem cells and human embryonic stem cells (ESC) (Lim et al. 2016).

A good conduit must be able to resist kinking and retain a constant diameter of the lumen when bent at wide angles and restore its original form when the bend was removed. The flexibility of the HUCA and HUCV were significantly decreased after crosslinked with 0.4% and 0.7% genipin, respectively, but they still resumed their original shape after it was bent for 5 minutes. This result showed that the genipin crosslinked artery and vein were resistant to luminal occlusion and have a high potential to maintain an open lumen when the conduit was sutured in joint areas.

Initially, it was expected that the clHUCA and clHUCV have increased mechanical strength compared to the native and decellularized group. However, the obtained results showed no significant increase in the mechanical strength of the clHUCA and clHUCV groups compared to the native and decellularized group. This could probably be due to the large standard deviation, the length of each specimen being too short and human error, such as the specimen was not centrally placed at the machine, which made it too hard when clamping both ends with a clamping steel. The variation could also be caused by the samples that was obtained from different donors, which has a high biological variation, and the variation was further expanded during the manipulation of the sample such as decellularization, crosslinking and incubations. In future studies, the variation should be reduced by selecting biological samples with similar physical properties before further manipulations were performed.

Conclusion

The decellularization and genipin crosslinking of human umbilical cord artery and vein were done successfully. The genipin concentration of 0.7% was sufficient to crosslink the conduit, which increased its degradation time. Genipin concentration of 0.7% proved to be the most biocompatible. Together with other suitable properties of the clHUCA and clHUCV, such as reduced swelling, flexibility, porosity and mechanical strength, clHUCA and clHUCV is suitable as a conduit for various tissue engineering applications, especially as nerve conduit. Further work to compare the genipin crosslinked HUCA and HUCV with autologous nerve conduit in rat model is warranted before this study can be translated to clinical setting.

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