Cytotoxicity and biocompatibility evaluation of chitosan-beta glycerol phosphate-hydroxyethyl cellulose hydrogel on adult rat liver for cell-based therapeutic applications

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Abstract: The pace of developing cell-based therapeutic systems by application of cellular scaffolds has been steady though slow. In present study, a chitosan based scaffold, CH-β-GP-HEC, was implanted into the rat liver to evaluate its biocompatibility, and particularly to test its cytotoxic effects during six months after implantation. The injected rats showed no obvious inflammatory responses during examination. Histological analyses revealed no difference between sections of the livers of test, vehicle and control groups after implantation, except regions which were occupied by injected scaffolds in the test group. The microscopic observations revealed that the size of the implanted scaffolds decreased by time. Moreover, analysing liver function based on the activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as biomarkers of liver injury, showed a significant increase in the first two weeks after implantation. This rate however, returned to normal level gradually. This reduction of the scaffold size along with the gradual reduction of the injury markers are signs of biodegradability and biocompatibility of the scaffold which make it a suitable candidate in cell based therapeutic programmes.

Keywords: AST; aspartate aminotransferase; ALT; alanine aminotransferase; CH-β-GP-HEC; chitosan; biocompatibility; cytotoxicity.

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1 Introduction

In the last decade cell based therapeutic approaches have provided encouraging promises for treatment of some incurable diseases. However, these promises have been shadowed by immune response, gene silencing and low maintenance of the implanted cells or concerns regarding their migration to other sites (Zhou et al., 2004; Engelhardt and Coisne, 2011; Gregory-Evans et al., 2012; Haddad-Mashadrizeh et al., 2013a; Haddad-Mashadrizeh et al., 2013b). Apart from immune response and other dilemma in these therapeutic methods, concerns about cell migration from the transplanted sites are one of the biggest challenges in these methods for clinical applications (Qiu et al., 2012; Huu et al., 2013). In this regard, the field of tissue engineering has expanded its attempts to achieve systems for cell confinement in transplantation sites (Molinaro et al., 2002; Ahmadi and De Bruijn, 2008; Huu et al., 2013) by using various synthetic or natural

scaffolds (Zitter and Plenk, 1987; Ozawa and Kasugai, 1996; Jin et al., 2009; Neshati et al., 2012). Hydrogels are a class of biomaterials, which are very similar to soft tissues due to their high water content, the mechanical properties (low modulus and elasticity), softness, oxygen permeability and excellent biocompatibility (Li et al., 2012). Among the natural hydrogel-based biomaterials, chitosan salts have been proposed as promising biomaterials in tissue engineering practices (Molinaro et al., 2002; Khor and Lim, 2003; Li et al., 2012). Chitosan possesses a wide range of properties that make it appropriate for tissue engineering and regenerative medical applications, including its biodegradability, biocompatibility, anti-bacterial and anti-fungal activity, wound healing properties and bioadhesive character (Rabea et al., 2003; Costa-Pinto et al., 2011; Naderi et al., 2011). In the last decade, an increasing number of *in situ* gel systems, based on chitosan and its derivatives have been investigated for various pharmaceutical and biomedical applications (Ruel-Gariepy and Leroux, 2004; Ishihara et al., 2006; Jin et al., 2009; Gao et al., 2012). Thus, chitosan, as a scaffold, could have critical roles in cell confinement by providing a suitable physicochemical and biological three-dimensional microenvironment for cell growth, differentiation and promotion of cell adhesion when encapsulated in the scaffold (Nakashima and Akamine, 2005; Willerth and Sakiyama-Elbert, 2008; Reilly and Engler, 2010; Gao et al., 2012). Although numerous studies have demonstrated that chitosan is a non-cytotoxic, biodegradable and biocompatible polymer, its derivatives should be carefully assayed before further applications. In this regard, our study is focused on the cytotoxicity and biocompatibility evaluation of a chitosan based scaffold, CH-β-GP-HEC hydrogel, on rat liver following its direct implantation into the organ. Liver is a key site for many pathways and numerous metabolic inherited diseases have their origin in this organ (Nguyen and Ferry, 2004). Millions of patients worldwide suffer from end-stage liver pathologies, whose only curative therapy is orthotopic liver transplantation (Fontana et al., 2002; Nguyen and Ferry, 2004; Kamada et al., 2009). However, this method is associated with numerous problems, including a chronic shortage of donors, high cost, rejection and side effects for the donor (Ochiya et al., 2010; Piscaglia et al., 2010; Dianat et al., 2013). To overcome these limitations, other alternative methods have been evaluated, including cell based therapies (Flohr et al., 2009; Ochiya et al., 2010; Piscaglia et al., 2010; Dianat et al., 2013). On the other hand, the liver represents one of the most important targets for gene delivery because of the ready access of the transgene product to the systemic circulation (Kren et al., 2002; Prieto et al., 2003; Nguyen and Ferry, 2004; Dai et al., 2006). Developing effective scaffolds which could be impregnated with desired cells can lead to enhanced survival, higher local retention and extended engraftment of transplanted cells at the injection site, as compared with standard injection techniques. Therefore, developing effective therapies based on combining proper scaffolds, cells and genes might help in relieving the suffering of many patients.

2 Materials and methods

2.1 Preparation of injectable CH-β-GP-HEC scaffold

Chitosan (CH) powder (Polysciences, Germany) with a molecular weight of 1000 kDa was sterilised by autoclave and left to air dry for at least 2 hours. 0.225 g of the powder was then dissolved in 9 ml of hydrochloric acid (0.1 M) with shaking. On the other hand, 2.25 g of β-glycerol phosphate (β-GP) (Sigma, Germany) was dissolved in 3.5 ml of

deionised water and sterilised using a 0.2 μm filter. Both solutions were chilled on ice for 15 min to avoid their gelation. The ice-cold GP-deionised water was then added drop wise to the ice-cold chitosan solution with continuous stirring to form a clear solution. Then, 0.125 g of hydroxyethyl cellulose (HEC) (Sigma, Germany) was dissolved in 10 ml of Dulbecco's modified Eagle's medium (DMEM) and added to CH-β-GP solution, immediately before injection. The final ratio of CH: β-GP: HEC in the final solution was 1.5% : 15% : 0.18%.

2.2 Experimental animals

In all stages of the study, we used two-month old Wistar rats with average weight of 250 g. Animals were kept in normal day-night cycle (12/12 hours), standard temperature (25 ± 2°C) and humidity conditions and fed by lab chow and tap water. All tests were carried out in accordance with guidelines of the Animal Care section of Ferdowsi University of Mashhad and approved by the University Animal Ethics Committee.

2.3 Transplantation procedure

Animals (n = 56) were divided into two groups of vehicle (DMEM, n = 7) and test (DMEM-Scaffold n = 7) and each group was examined at four different time points (15, 45, 90 and 180 days) after implantation. Three rats without any treatments were also used as controls at each time point. Briefly, each animal was anesthetised by intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine hydrochloride (5 mg/kg). Then, after laparotomy under aseptic conditions, approximately 200 μl of DMEM or DMEM-Scaffold, were slowly injected into the Left Lateral Lobe (LLL) of the liver of each case using a 30 gauge insulin syringe. No immunosuppressants were used in these expriments. The incision was then closed with the silk suture and 50,000 units of penicillin/kg body weight were injected intramuscularly. After recovery from the surgery, the animals were returned to their cages and assessed at different time points up to 6 months.

2.4 Histological analysis

The examined rats were re-anesthetised at different time points and sacrificed by cardiac perfusion with 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) until the outflow became clear. The abdomen was entered through a midline incision, and the LLL of the liver was dissected out and immediately postfixed in 4% paraformaldehyde for at least 24 hours. The samples were then embedded in paraffin and a rotary microtome (Leitz, Australia) was used to prepare 5 μm sections of the liver. Slides were examined under a light microscope (Olympus AH3-RFCA, Japan) after staining by Hematoxylin/Eosin (H/E).

2.5 Assessment of AST and ALT activity

The hepatotoxicity due to scaffold implantation was verified by measuring the activity of Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) changes. To do so, 5 ml blood was collected from portal vein of the liver immediately before cardiac

perfusion. Blood sera were stored at –20°C until tested. Liver enzymes, including AST and ALT were evaluated by a colourimetric method using ASAT (GOT) kit (Pars Azmon, Iran).

2.6 Statistical analysis

All data are expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to analyse the activity of enzymes over time. Analysis of variance was done by a Tukey post-hoc test. Statistical significance was considered at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$. All statistical analyses were carried out in triplicate with SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

3 Results

3.1 Assessment of the scaffold biodegradability and biocompatibility

As shown in Figure 1, the interface regions occupied by the scaffold constructs are detectable in the sections of the liver tissues in each experiment up to 180 days post-implantation; nonetheless their dimensions are reduced by time. Moreover, the microscopic observations showed that the injection of the scaffolds into the liver resulted in no morphological changes as revealed by H/E staining (see Figure 2).

Figure 1 Histological examination of gradual biodegradation of a chitosan based scaffold, CH-β-GP-HEC, in adult rat livers during 6 months after implantation by Hematoxylin/Eosin staining. A, B, C and D represent samples of liver sections at different time points of 15, 45, 90 and 180 days post-implantation, respectively. As shown in the figure, the areas occupied by the scaffolds (pointed by arrows) are significantly reduced by time which indicates biodegradation of the scaffold

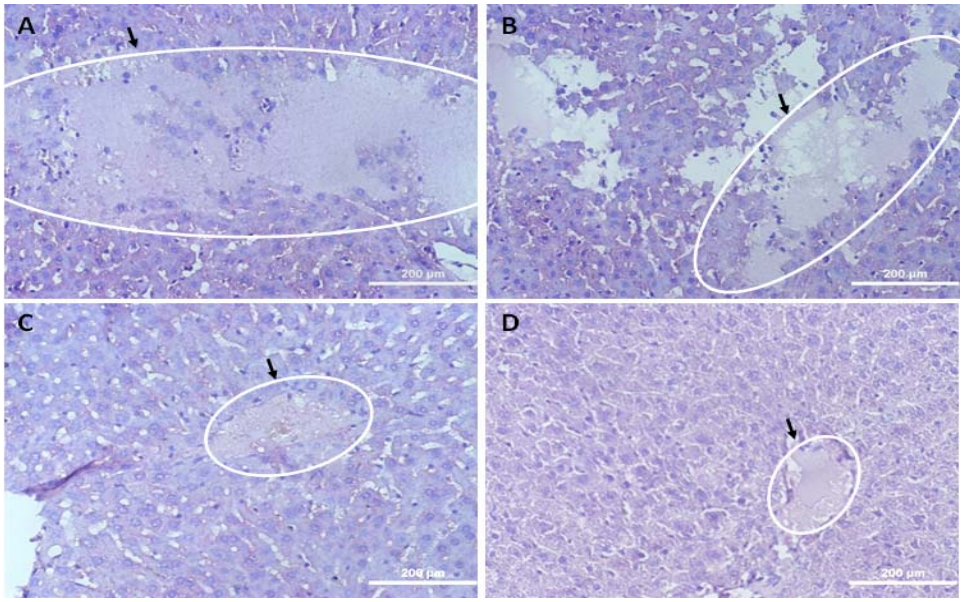
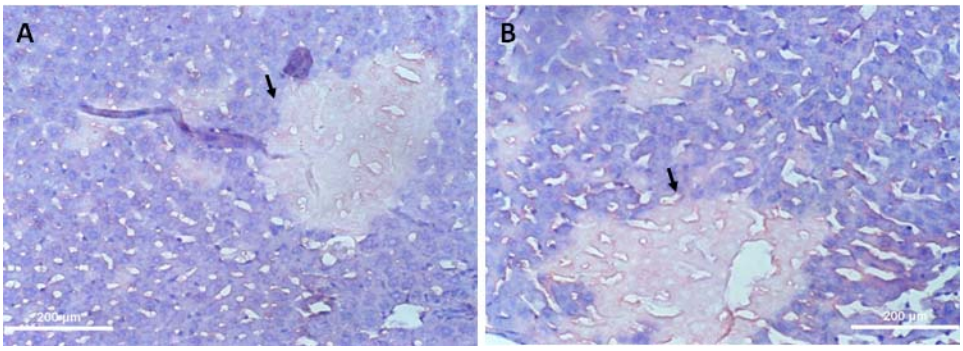


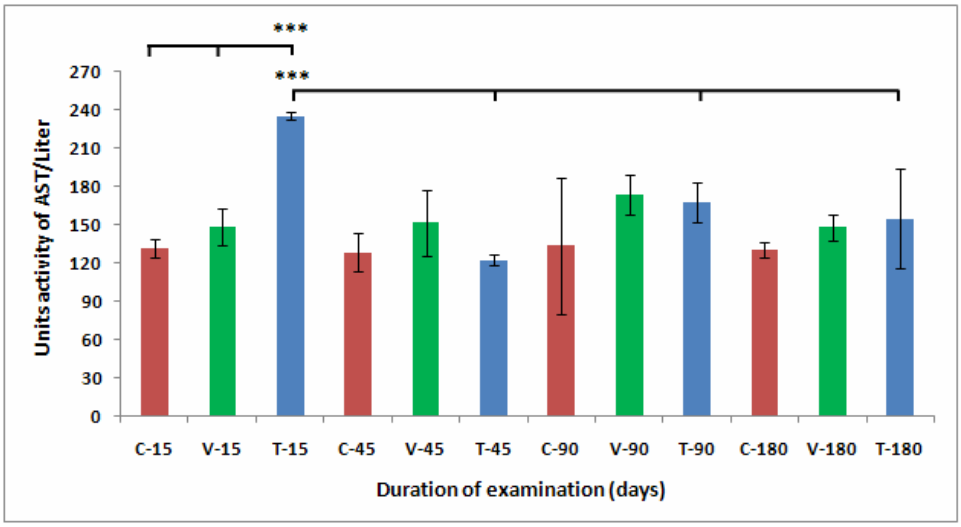
Figure 2 Micrographs representing penetration and harmonisation (pointed by arrows) of chitosan based scaffold, CH-β-GP-HEC, into the rat liver tissues, at different time points of 15 (A) and 45 (B) days post-implantation. As shown in this figure, no histological changes can be observed in the liver tissue as indicated by Hematoxylin/Eosin staining



3.2 AST activity assay during six months after implantation

AST activity, as a biomarker of liver injury or evidence for scaffold hepatotoxicity was assayed during 6 months after implantation. Our analysis showed a significant increase ($P \leq 0.001$) in AST activity at day 15 after the scaffold implantation in comparison to controls (see Figure 3). However, this activity was decreased by time in a way that from 45 days after implantation, there was no significant difference in AST activity in the test group as compared to the vehicle and control groups.

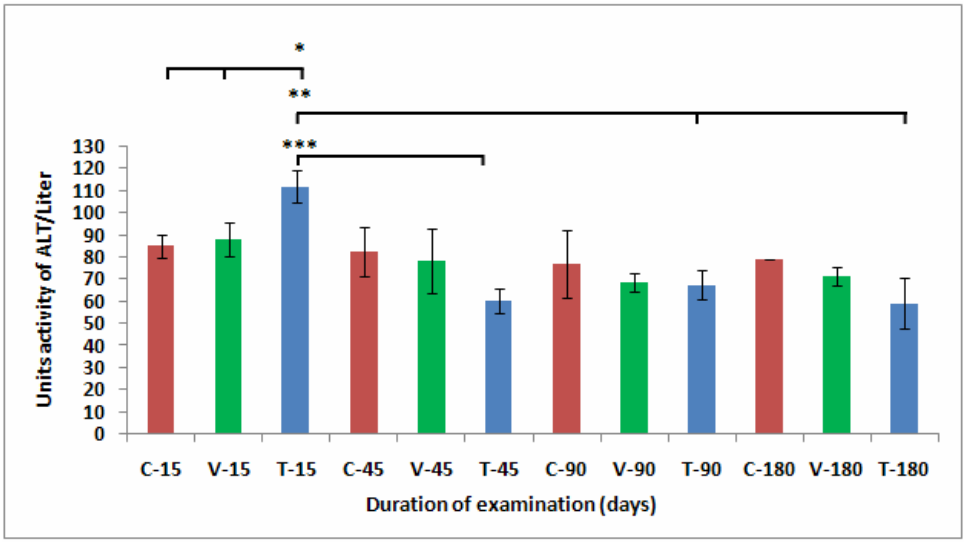
Figure 3 Analysing the aspartate aminotransferase activity in the blood following CH-β-GP-HEC scaffold implantation into the liver. Control, vehicle and test groups are indicated by C, V and T, respectively, followed by digits corresponding to the number of days after implantation. Data are expressed as means ± SD. *** represents $P \leq 0.001$ (see online version for colours)



3.3 ALT activity assay during six months after the scaffold implantation

ALT activity, as another biomarker of liver injury was also assayed in this study. As shown in Figure 4, the activity of this enzyme, similar to AST, showed a significant increase ($P \leq 0.05$) at day 15 after implantation as compared to the vehicle and control groups, and it started to decrease by time.

Figure 4 Analysing the alanine aminotransferase activity in the blood following CH-β-GP-HEC scaffold implantation into the liver. Control, vehicle and test groups are indicated by C, V and T, respectively, followed by digits corresponding to the number of days after implantation. Data are expressed as means ± SD. ***, ** and * represent $P \leq 0.001$, $P \leq 0.01$ and $P \leq 0.05$, respectively (see online version for colours)



4 Discussion

Combining stem cells with biomaterial scaffolds provides a promising strategy for tissue engineering and cell therapy (Willerth and Sakiyama-Elbert, 2008). Use of biodegradable polymers has become widespread (Nguyen and Lee, 2010; Jayakumar et al., 2011; Wang et al., 2012), but the cytotoxicity and biocompatibility of the scaffolds must be tailored before their clinical applications. In this regard, several injectable biomaterials such as collagen gel (Wakitani et al., 1989), calcium alginate (Paige et al., 1996) and fibrin glue (Yamada et al., 2003) have been developed. These scaffolds have several advantages, for example they are able to fill any space or shape of a defect site, cells and therapeutic agents can be incorporated within the solution prior to the injection, and more importantly, the systems can be implanted into the site without surgery. However, there are several inherent disadvantages with these particular materials including a variable degradation rate, an inadequate tissue penetration and adverse host immune responses,

which have been surmounted by chitosan based scaffolds (Molinaro et al., 2002; Kim et al., 2008a; Costa-Pinto et al., 2011). Some of these properties were tested in current study by implantation of a thermosensitive chitosan based scaffold, CH-β-GP-HEC, into the adult rat liver. Reduction in the size of the scaffold constructs which was observed by time (see Figure 1) clearly supports the idea of biodegradability of the CH-β-GP-HEC scaffold in the liver. This result is consistent with previous reports, showing the biodegradability of other chitosan based scaffolds after implantation into various tissues (Nguyen and Lee, 2010; Costa-Pinto et al., 2011; Li et al., 2012; Wang et al., 2012). Natural polymers such as chitosan have been described as biocompatible and biodegradable with tailorable degradation rates (Costa-Pinto et al., 2011). The degradation rate of chitosan is inversely related to the degree of deacetylation, which represents the proportion of *N*-acetyl-glucosamine units to the total number of units (Chatelet et al., 2001). Moreover, our histological observations revealed that the scaffold constructs could penetrate into the liver tissue without causing any morphological changes (see Figure 2), which also proves the biocompatibility of the CH-β-GP-HEC scaffold, consistent with other reports (Kim et al., 2008a; Willerth and Sakiyama-Elbert, 2008; Costa-Pinto et al., 2011). On the other hand, it has been shown that the levels of serum AST and ALT activity are critical in the diagnosis and assessment of liver diseases and they are recommended for the analysis of hepatocellular injury as highly sensitive and fairly specific preclinical and clinical biomarkers (Carakostas et al., 1986; Boyd, 1988; Sherman, 1991; Travlos et al., 1996; Kim et al., 2008b; Ozer et al., 2008). Therefore, the activity of these biomarkers of liver injury, AST and ALT, were assayed and the results indicated a significant increase at the first two weeks after scaffold implantation, as compared to the vehicle and control groups; nonetheless, these activities reduced and returned to the control level by time (see Figures 3 and 4). These results could reflect possible damage to hepatocytes after scaffold implantation. However, these slight increases in the activity of AST and ALT and their return to the normal level by time could be due to trivial hepatic injury or even the damage to the abdominal muscles during laparotomy. It has been demonstrated that muscle injury and also procedures related to handling, as extrahepatic factors, can cause increase in serum transaminase activity of AST and ALT, but AST is generally higher than ALT when both are concurrently increased (Swaim et al., 1985; Valentine et al., 1990; Boone et al., 2005), which is consistent with our data (see Figures 3 and 4). On the other hand, an increase in serum ALT activity in the range of 2–4 times or higher as compared with controls should raise concern as an indicator of potential hepatic injury unless a clear alternative explanation is found (Boone et al., 2005). Therefore, slight increases in the activity of AST and ALT could be related to extrahepatic factors instead of side effects of scaffold implantation. In conclusion, our findings are consistent with former reports about biodegradability and biocompatibility of chitosan based scaffolds which led to no obvious inflammatory responses after surgery up to at least 6 months. So, this scaffold can be a suitable candidate for cell based therapeutic methods, which may lead to extended engraftment of transplanted cells at the injection site, especially in liver disorders. However, the potency of this scaffold in cell confinement and local retention should be evaluated after impregnation with desired cells and also a more detailed analysis should be carried out to verify the outcome.

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