

Obtaining of Hydrogels using PVA and HEC for Adipose Tissue Regeneration

Karla L. Tovar C¹, Genaro Tamayo¹, Alejandro Donohue², Takaomi Kobayashi³, Rosa A. Saucedo A^{1*}

¹Biomedical Institute, Universidad Autonoma de Ciudad Juarez, Mexico

²Clinic and Breast Surgery, Hospital Angeles, Mexico

³Department of Materials Science and Technology, Nagaoka University of Technology, Japan

Abstract

Polyvinyl Alcohol (PVA) and Hydroxy Ethyl Cellulose (HEC) were used to prepare hydrogel for tissue regeneration. Female rabbits were used to evaluate the obtained hydrogels for the regeneration of adipose tissue. Mechanical and biocompatible properties were evaluated. AFM (Atomic Force Microscopy) and SEM (Scanning Electron Microscopy) showed the roughness around 5.447 nm and pore size from 1 to 7.9 μm . *In vivo* tests were conducted during two years in female rabbits. Histological images showed stable fat formation over long-term without adverse reaction or necrosis. The obtained results indicated that PHEC30 hydrogel may provide a viable approach for the regeneration of adipose tissue in female rabbits as first step as an alternative solution for women who suffered a radical mastectomy.

Keywords: Polyvinyl alcohol; Hydroxyethyl cellulose; Hydrogel; Adipose tissue

Introduction

Advances in study of materials for medical applications rely on enhanced understanding of their properties both surface and bulk. Among diverse candidate, hydrogels, three dimensional swollen macromolecular networks offer unique possibilities to engineer materials with properties closely matching human tissue, especially with regard to mechanical properties, water content, and accessibility to solutes [1-3]. While chemically cross-linked gels dominate the field, association of polymers through non-covalent linkages, i.e. physical hydrogels, is friendlier toward fragile biological cargo and is therefore highly attractive for biomedical applications. Among these, physical hydrogels based on poly (vinyl alcohol), PVA, stand out due to their superior mechanical properties and biocompatibility. These materials have a well-documented history of successful applications in biotechnology and biomedicine, especially in enzyme and whole cell immobilization, biomass conversion and tissue engineering. Today, tissue engineering provides numerous biomaterials as structural components to improve or replace biological functions, for example improving quality of life of cancer patients. In this manner, life style of breast cancer patients changed dramatically after mastectomy, surgery procedure to remove breast tissue [2-9]. Actually, the number of diagnosed women with breast cancer that suffered a radical mastectomy is increasing day by day. This surgery affects dramatically the auto image of women. On the other hand, reconstructive surgery offers only fat rotation and use of silicone implants to solve the problem. For these, the necessity of an alternative for breast cancer patients is evident. Currently, tissue regeneration provides numerous materials as structural components to promote tissue regeneration. These options, commonly involve seeding pre-adipocyte cells on polymer scaffolds as poly (L- lactic-co-glycolic) acid (PLGA), collagen, Hyaluronic Acid (HA) Polyethylene Glycol (PEG) and gelatin [3-10]. In this way to obtain a successful scaffold, the material should be biocompatible, absorbable, and its surface should interact with cells and tissues to facilitate large volume soft tissue regeneration, and promote its vascularization [1-11].

Moreover, several strategies had been tested to promote adipose regeneration including cell seeding and direct use of adipose tissue (omentum) [12-18]. In 2004, Masuda et al. used directly omentum to induce the formation of adipose tissue *in vivo* with good results by

a short time period [19]. Other research consisted on the co-culture of adipocyte and endothelial cells, trying to induce the formation of vessels, or in another case, the revascularization of the implanted material with the vessel from host tissue growing into the tissue site [20-22]. All these strategies showed good results but only in short time, but all the approaches showed problems to obtain a stable fat formation by long-term. For this reason, until today researchers focus their attention to obtain stable fat formation by long-term [23, 24].

As seen in literature, due to their low toxicity and biocompatibility, PVA and HEC are better candidates to prepare materials for medical applications. In addition, hydrogels prepared with PVA showed good results in cell adhesion and tissue growing as well as applied as wound dressings, fillers, and tissue regeneration [25-30]. In the present work, we report the obtaining of PVA/HEC hydrogels for adipose regeneration. We focus in the study of the properties of the obtained hydrogels *in vivo* and *in vitro*. Adipose regeneration in several hydrogel was studied in short-term and long-term [31-34].

Experimental Procedure

Materials

Poly Vinyl Alcohol (PVA), Hydroxy Ethyl Cellulose (HEC), both purchased by Aldrich. Glutaraldehyde was purchased by Aldrich. Hydrochloric acid (HCl), purchased by Alpha Assar. Paraformaldehyde and Para film by Aldrich. Hematoxylin- Eosin by Aldrich.

Hydrogel preparation

The HEC and PVA hydrogels were prepared by dissolving HEC and PVA to obtain polymeric solution of 5 wt%. PVA concentration

*Corresponding author: Saucedo Acuna Rosa A, Biomedical Institute, Universidad Autonoma de Ciudad Juarez, Anillo envolvente del Pronaf y Estocolmo s/n. CP 32300, Cd Juarez, Chih, Mexico, Tel: +52-656-688-18-00; E-mail: rosasaucedo@yahoo.com.mx

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was varied from 10 wt% to 90 wt% in the polymeric solution. PVA and HEC were dissolved in water and stirred at 80°C for 60 min. Glutaraldehyde was used as cross linker in acid environment. After 2 h the resulting solution was casted into a glass plate to obtain a thin film. Then, the resulting films were washed with water by soxleth system for 3 h at 80°C. Finally, films were dried at 37°C for 72 h. Before surgery, the dried hydrogel films were submerged in distilled water for 30 min.

Evaluation of the obtained hydrogel films

Equilibrium Water Contents (EWC) of the resultant hydrogel films were determined by weighting the wet and dry samples. Samples (5 x 5 mm²) were cut from casted films. Samples were dried with vacuum and kept in desiccator until the measurement. The samples were then swollen in distilled water at 37°C for 36 h. Then, samples were removed and wrapped with filter paper to remove excess of water. The weight of hydrated samples was then determined. The percent EWC of hydrogels was calculated based on $EWC = ((W_h - W_d) / W_d) \times 100$, where the values of W_h is the weight of the hydrated samples and W_d is the dry weight of the samples. For each specimen, four independent measurements were determined and averaged.

For the contact angle measurements, three dried samples of the resultant hydrogel films were measured using a contact angle goniometer (Kyowa Interface Science) at room temperature. Samples (2 cm x 2 cm) were placed on a glass slide and mounted on the goniometer. A total of 3 µL of double distilled water was dropped on the air side surface of the films. The contact angle was measured after 10 s passed from the droplet process. At least eight were averaged to obtain a reliable value.

For measurements with Atomic Force Microscopy (AFM), samples (2 cm x 2 cm) were dried under vacuum overnight and images were recorded using Nanopics 100, NPX 100 (Seiko Instruments Inc. Tokyo Japan). For measurement of Scanning Electronic Microscope (SEM), after the hydrogel samples were dried, samples were coated with a gold layer. The SEM images were recorded by using JSM-5310LVB (JEOL, Japan) with a magnification of 5000 in magnitude.

The tensile of dried samples were carried out using hydrogel samples (50 mm x 10 mm) on a LTS-500N-S20 (Minebea, Japan), universal testing machine equipped with a 2.5 KN cell. Strips with a length of 50 mm and a width of 10 mm were cut from casted films with a razor blade. One set of samples which ruptured near mid-specimen length were considered for the calculation of tensile strength. Stress-strain curves were obtained during the measurement.

The value of the tensile strength (N/mm²) was calculated with the following equation:

$$\text{Tensile strength} = \text{Maximum Load} / \text{cross section area}$$

The viscoelasticity of swelled hydrogel films with 2 cm of diameter and having 5 mm of thickness was carried out at 37°C, under 1.33 N and a frequency of 100 rad/s using a Physical MCR 301 equipment of Anton Paar. FT-IR spectroscopy was applied to examine components by using FT-IR 4100 series (Jasco Corp, Japan).

Adipose tissue regeneration in female rabbits

Following the research and bioethics council of The Autonomous University of Juarez for use and care of laboratory animals, we used in these trails New Zealand female rabbits, three months old, and with a weight of 3.5 Kg. The selected surgical area was located in the abdominal breast, left lateral side. Before the surgical procedure, 2 cm

conic shape hydrogels were swelled with (?). Approximately 2 cm² of omentum was used to cover the conic shape hydrogel and it was placed between muscle and subcutaneous tissue. Subsequently, the evaluation of the female rabbits was done each week registering weight and condition of surgical area.

Histological analysis

After 2 months and 2 years biopsies of the new tissue formed were taken in the area around the embedded hydrogel. A cross-section of the biopsy was fixed overnight in 4% of paraformaldehyde and then embedded in paraffin. In addition, sections of 8µm of thickness were prepared. According with a standard procedure [20, 22] Hematoxylin-Eosin was used to observed the morphologic details of the new tissue formed.

Results and Discussion

Evaluation of the obtained hydrogel films

Table 1 lists properties of the obtained hydrogels. The content of HEC was varied from 90 wt% to 10 wt%. The samples were named depending of HEC content, as showed in (Table 1). In the case of water content measurements, the hydrogel samples were immersed in distilled water and reached to the equilibrium after 36 h. Figure 1 shows pictures of PHEC30 in (a) dried and (b) wet conditions. Water content value decreased from 727% to 205% with the decrease of HEC in the hydrogel sample. As shown in the obtained values of contact angle, it was observed a tendency related to HEC content. At higher content of HEC, water molecules were capable to penetrate and interact easily into the hydrogel film. It was notice that the hydrogels with higher content of HEC had very soft and flexible shape [34, 36]. For the uniaxial tensile testing, the samples (10 mm x 50 mm) were placed between two clamps in dried conditions. Table 1 shows tensile strength and elongation values for the hydrogel films. In the case of PHEC90 and PHEC10,

Sample Name	PVA (wt%)	HEC (wt%)	Thickness (mm)	EWC (%)	Contact angle (°)	Tensile strength (N/mm ²)	Elongation (%)
PHEC90	10	90	0.12	727	56	38	2.23
PHEC80	20	80	0.10	642	61	67	1.97
PHEC70	30	70	0.15	536	63	86	1.78
PHEC60	40	60	0.10	479	65	102	1.44
PHEC50	50	50	0.16	379	67	109	1.35
PHEC40	60	40	0.14	301	68	122	1.33
PHEC30	70	30	0.11	251	69	138	1.08
PHEC20	80	20	0.13	240	71	183	1.04
PHEC10	90	10	0.15	205	74	228	0.63

Table 1: Properties of the Phec Hydrogel Films Varying the Content of Hec.

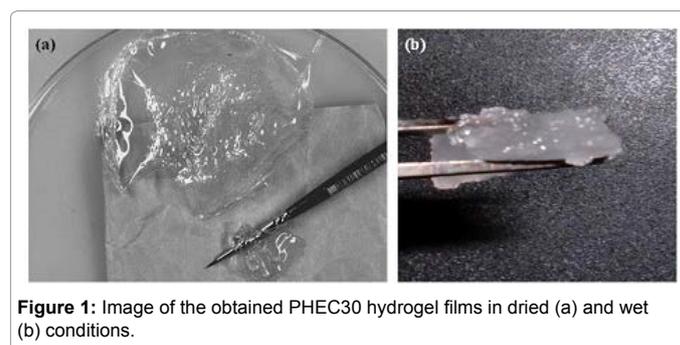


Figure 1: Image of the obtained PHEC30 hydrogel films in dried (a) and wet (b) conditions.

the tensile strength values increased from 38 N/mm² to 228 N/mm² and the elongation decrease from 2.23% to 0.63% respectively. This might be due to the presence of HEC in the hydrogel network, which could diminish the interaction between PVA and the cross linker. This could explain the decrease of the stiffness of the films prepared with higher content of HEC. The observed higher tensile value in the case of PHEC10 could be attributed to the higher interaction of PVA with the glutaraldehyde used as cross linker and to the lower content of HEC. It has been also postulated that chemical structure of HEC allows better interaction with water molecules by the dissociation of the OH groups.

Moreover, Figure 2 shows the stress-strain curves of thirty samples for each PVA percentage at room temperature with a thickness ~0.2 mm. In Figure 2 it can be notes that the chain in the network could not keep strands from moving away from their relative position. Due to the test specimens being soft, a low concentration of cross linker used as required for the regeneration of adipose tissue. Yanxia and coworkers reported in their cyclic tests at low levels of strain amplitude (<1 x 10⁻³) and at frequencies below 100 Hz that adipose tissue follow a linear visco-elastic solid behaves showing a compressive storage modulus of 2 kPa and a compressive loss modulus of 0.5 kPa [18]. In other words, the mechanical properties of the PHEC30 hydrogel are similar to the properties of the adipose tissue showing only an increase of 10% in the viscosity. These properties support the successful results of the trials *in vivo* followed by histopathology and microscopy images. In addition, according with Lutolf et al., a representative curve of stress versus strain, the adipose tissue has a Young's modulus of 1 kPa at a strain rate of 0.002 s⁻¹ (at low strain rate), whereas at strain rates of 1000 s⁻¹ the modulus increases by more than three orders of magnitude with values of 3 MPa [17].

Furthermore, to evaluate the surface of the obtained hydrogel films AFM measurements were carried out in dried conditions. Figure 3 shows AFM images of hydrogel samples (20 mm x 20 mm). Figure 2 shows AFM images of hydrogels surface of (a) PHE10, (b) PHE70 and (c) PHE90. Significant difference in roughness (from 7.89 to 2.87 nm) of the obtained samples as observed. The measurements strongly suggest diminish of roughness of the hydrogels when HEC content increased. Moreover, the obtained AFM images revealed that the addition of HEC changed the surface of the hydrogel. It has been postulated that roughness is an important factor in biocompatibility

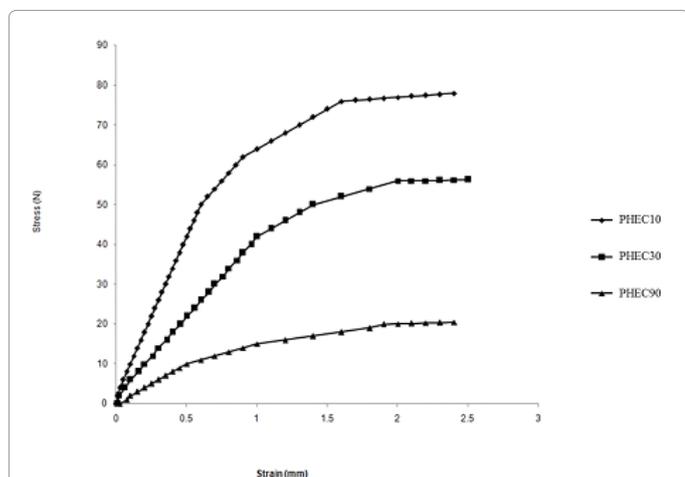


Figure 2: Stress-strain curves for each HEC percentage in the obtained hydrogels films at 37°C.

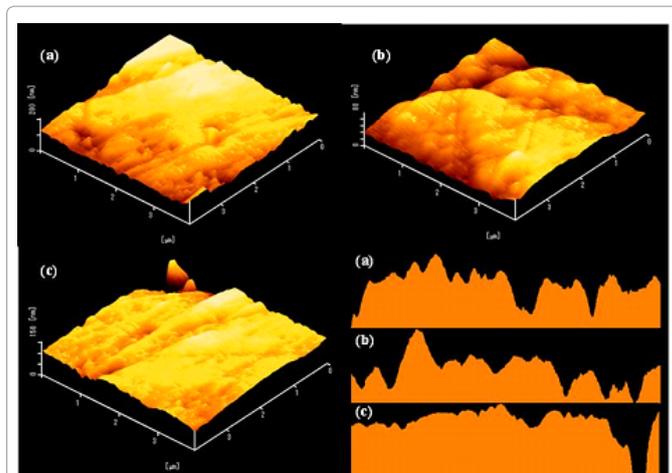


Figure 3: AFM images of hydrogels film surface. The amount of HEC was varied in samples PHEC90 (a), PHEC70 (b) and PHEC10 (c). Roughness of the hydrogels is showed in right corner of the Figure for PHEC90 (a), PHEC70 (b) and PHEC10 (c).

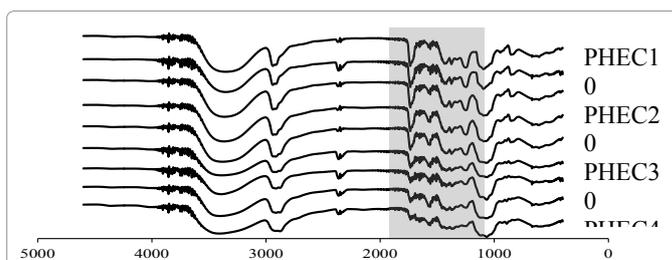


Figure 4: FT-IR spectra of the hydrogel films in dry conditions prepared with different HEC 14 content.

providing a suitable environment for protein adsorption. A diminish in surface roughness could affect protein adsorption and further cell adhesion [35, 36].

Figure 4 shows the infrared spectra of the hydrogel films obtained varying the concentration of HEC in dry conditions. The FT-IR spectra shows strong peaks around of 3550 and 3200 cm⁻¹ linked to O-H band stretching, while the predominant peak around 1651 and 1637 cm⁻¹ refers to C = O stretching of carboxylic groups of the HEC. In addition, peaks around 1150, 1160, and 1120 assigned to C-OH, C-O-C, and C-C bands, as well as bands showed around 1059 and 1035 cm⁻¹ related to CH₂ and OH groups, respectively [37]. Moreover, the peaks in the range of 1300-1700 cm⁻¹ correspond to the characteristic peaks of PVA [38]. It was noticed that the intensity of these peaks increase with the decrease of the HEC content in the film. This could explain the difference in the interactions between the crosslinker and the polymers affecting the mechanical properties of the obtained films.

Besides, in order to determine the properties of the polymeric matrix offered by the obtained hydrogel films, electronic image of microscopy of dried samples were obtained. Figure 5 shows SEM images of dried surface of PHEC 30, where porous exhibit a size of 1 to 7.9 μm. Also, diminish of pore size was observed with the increment of HEC content in the obtained hydrogel film. This could be attributed to the effect of the HEC on the mechanical properties showed in Figure 2. It has been found that pore size of the polymeric matrix plays an important role in tissue regeneration applications [35, 36]. The obtained results suggested that pore distribution permits the

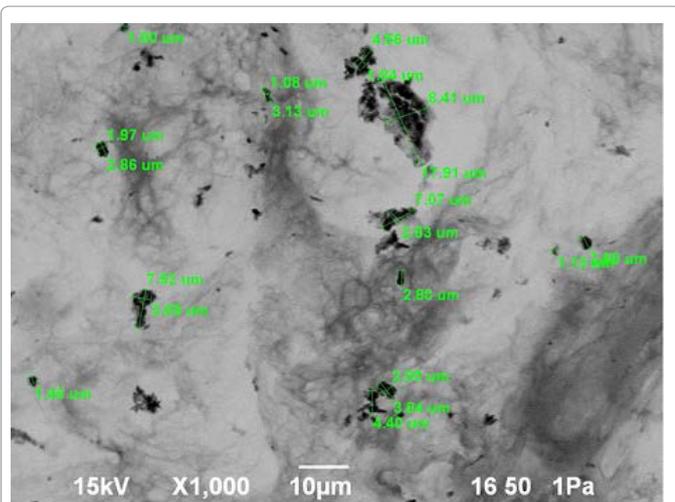


Figure 5: SEM Image showing the distribution of porous in PHEC30 hydrogel.

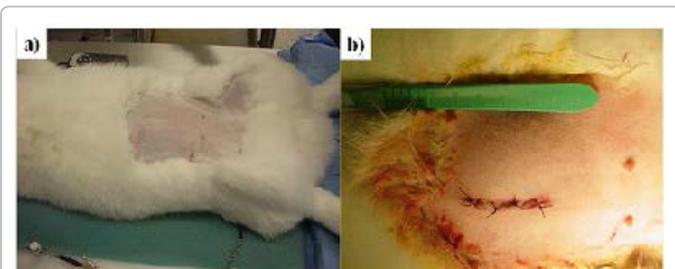


Figure 6: Images showing the surgical area before the implantation (a) and after the implantation of the hydrogel (b). The surgical procedure was made in New Zealand female rabbits, three months old and with a weight of 3.5 Kg.

deposition and growing of different kind of cells making possible the formation of neovascularized fat.

In Vivo hydrogel assays in female rabbits

Furthermore, Figure 6 shows images of the surgery section before and after procedure. Before the surgery the hydrogel sample needs to be swelled in order to consider the volume of the hydrogel at maximum swelling point for its manipulation during the surgery. In figure 6a we can see the selected area for the surgery, and figure 6b shows the volume formed when the fragmented omentum was involved by the hydrogel immediately after the surgery. The obtained volume showed in Figure 6b remain with the time. It was observed that after weeks passed the volume increased and more defined round shape was found, as showed in Figures 7 a and 7 b. Two months after the surgery, the weight of the rabbit increased to 0.68 kg and the abdominal volume was 70 ml, as showed in Figure 7a. Figures 7c and 7b showed histological images after two months and two years of the surgery, respectively. Figure 7c shows light gray portions related to the gel settled in the rabbit. In the other hand, Figure 7d shows remarkable diminish of gel. After two years, few gel surrounded of normal mature adipose tissue and few bands of stromal tissue was observed. It can be notices that the volume showed in Figure 7a and 7b did not showed significance differences, Figure 7d shows that the maintained volume is due to the formation of adipose tissue in the surgery section meanwhile the polymeric matrix was degraded. This could be attributed to the characteristics of obtained hydrogels. The spaces between the cross-linked polymeric chains form the porous where the tissue grown. The porous size increase its size

when the tissue grows, then the polymeric chains were separated and adsorbed losing the shape and properties of the polymeric matrix resulting in the diminish of the gel settled portion during the surgery.

It was observed that the new tissue grows following the natural shape of the body, for this reason we expected to find these reminiscences of hydrogel near to the extremes of the new adipose tissue. In Figure 8a it was observed a hydrogel portion surrounded by new adipose tissue. Moreover, Figure 8b shows excellent interaction between the hydrogel and the conjunctive tissue, this gives evidence of the cyto and biocompatibility of the prepared hydrogel film which was used for the body as a natural connector with the new tissue. Figure 8b also shows evidence of not adverse reaction between the hydrogel and the surrounded tissue, and bio integration as well as vascularization of the new tissue, our main purpose [20, 22]. In addition, Figure 8b shows the formation of new blood vessels, adipose transformation and occasional presence of multinucleated cells by reaction of foreigner body. Not evidence of necrosis or calcium deposits was founded [20]. Moreover, the weight of the female rabbits was varied to see the development of the regenerated tissue. In order to force the rabbits to lose weight, their food portion was reduced half during one week, reporting a weight and volume reduction of 0.227 kg and 20 ml respectively. On the other hand, to gain weight food ration was allowed unrestricted demand for one week, after this recovered of weight and

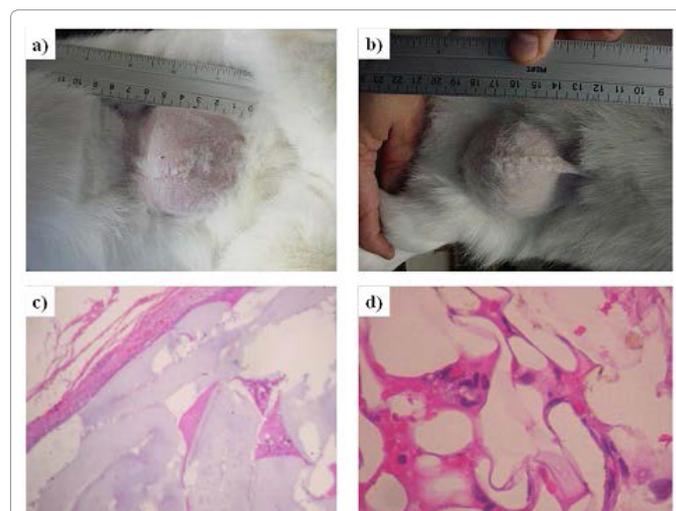


Figure 7: Images of the surgical area after 2 months (a) and 2 years (b), respectively. Pathology images of the tissue formed around the implanted scaffold after 2 months (c) and 2 years (d) after the surgery. The images showed the obtained results using the hydrogel PHEC30.

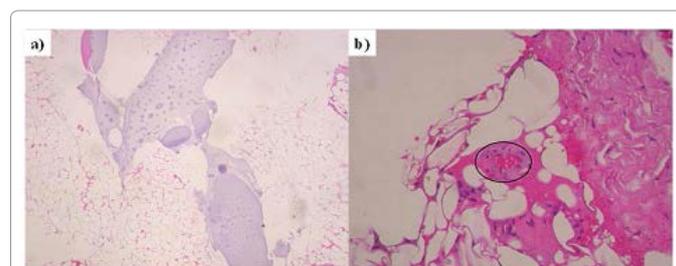


Figure 8: Pathologic images of the adipose tissue formed around the implanted hydrogel after 2 months (a) of the surgery and after 2 years of the surgery (b). New Zealand female rabbits were used, three months old and with an initial weight of 3.5 Kg before the surgery. The zone indicated with a black circle indicates cells forming a new vessel.

volume was observed. After two years, rabbits showed stable weight and abdominal volume.

Conclusions

Hydrogel films prepared with PVA and HEC were obtained using glutaraldehyde as cross linker. The hydrogel films prepared with higher content of PVA showed higher mechanical properties. It was proved that the PHEC30 film was capable to stimulate and directional the growing of adipose tissue due to its mechanical and biocompatible properties when the material was implanted *in vivo*.

Moreover, the roughness, porosity and mechanical properties of PHEC30 hydrogel promote excellent adherence and proliferation of surrounded tissue, allowing the formation of a stable volume of adipose tissue with excellent neovascularization.

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