Comparative analysis of preparation of human hepatocytes by the ethylenediamine tetraacetic acid and collagenase technique[1]

Estudo comparativo da obtenção de hepatócitos humanos pela técnica ácido etilenodiaminotetracético e da colegenase

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ABSTRACT
Purpose: To compare the viability of human hepatocytes dissociated by the ethylenediaminetetraacetic acid and collagenase techniques. **Methods**: Hepatocytes were prepared by dissociation of liver fragments obtained from hepatectomies performed for therapeutic purposes at the Service of Digestive Tract Surgery, Federal University of Triângulo Mineiro. **Results**: During the first 4 days of the experiment, 70% of the cells presented birefringent membranes and were not stained with 2% erythrosine, and were therefore considered to be viable. During the first 3 days, hepatocyte viability was on average 71% in the EDTA group and 76% in the collagenase group, with no significant difference between groups. No significant difference was observed between groups at any time. The secretion of albumin by the cultured hepatocytes was preserved up to the seventh day. Mean albumin secretion during the first 3 days was 50 µg/ml in the two groups and a reduction of albumin production was observed from the fourth to the seventh day. Again, no significant difference was observed between groups at any time. **Conclusion**: Cell viability and preservation of albumin secretion by hepatocytes are similar for the EDTA and collagenase techniques.
Key words: Cell Separation. Cell Transplantation. Edetic Acid.

RESUMO
Objetivo: Comparar a viabilidade dos hepatócitos humanos dissociados pelas técnicas do ácido etilenodiaminotetracético e da colagenase. **Métodos**: Hepatócitos foram preparados pela dissociação de fragmentos de fígado, provenientes de hepatectomias realizadas com o objetivo terapêutico no Serviço de Cirurgia do Aparelho Digestivo da Universidade Federal do Triângulo Mineiro. **Resultados**: Detectou-se que nos quatro primeiros dias de experimento 70% das células estavam com suas membranas biorrefringentes e não se coravam pela eritrosina a 2% portanto foram consideradas viáveis. Observou-se que nos três primeiros dias a viabilidade dos hepatócitos foi em média 71% no grupo EDTA e 76% na colagenase, diferença esta sem significado estatístico entre os grupos. Em nenhum momento, detectou-se diferença estatística entre os grupos. Com relação a preservação da secreção de albumina pelos hepatócitos em cultura observou-se que foi mantida até o sétimo dia. Da mesma forma, notou-se que nos três primeiros dias a média de secreção de albumina de ambos os grupos foi de 50 ìg/dl e que após o quarto dia verificou-se redução da produção até o sétimo dia. Também não foi observado diferença significativa em nenhum momento entre os grupos. **Conclusão**: A viabilidade celular e a preservação da função de secretar albumina pelos hepatócitos são semelhantes pelas técnica do EDTA e da colagenase.
Descritores: Separação Celular. Transplante de Células. Ácido Edético.

Introduction

Several research centers have been searching for auxiliary liver transplantation techniques either for definitive use in chronic or temporary situations or for acute situations, thus prolonging the waiting time for functional improvement of the regenerated liver or for appearance of a compatible liver transplant donor, with the latter certainly continuing to be the only therapy for end-stage hepatic insufficiency and metabolic disorders. The great expectation of finding a bioartificial liver has directed research at the utilization of isolated viable hepatic cells with preserved functions [1,2,3]. Sun[4], using a semipermeable alginate-polylysine membrane for the

microencapsulation of hepatocytes, reported satisfactory viability of the cells *in vitro* and *in vivo*[5]. Several investigators studying the preservation of hepatocyte function in homozygous Gunn rats noted a significant reduction of bilirubin levels over a period of 30 days when intraperitoneally transplanted microencapsulated hepatocytes were used [6]. Immunoprotection offered by the microcapsule membrane, as well as a significant reduction of bilirubin levels, has been demonstrated in Gunn rats receiving alginate-polylysine-microencapsulated hepatocytes [6]. The most commonly used methods for the dissociation of hepatocytes from other types of cells (biliary cells, supporting cells, vascular cells, etc.) without compromising their metabolic functions are EDTA (ethylenediamine tetraacetic acid) or collagenase perfusion, or a combination of both. Therefore, the objective of the present study was to compare the viability of human hepatocytes dissociated by the EDTA and collagenase techniques.

Methods

To compare the dissociation of hepatocytes by the EDTA and collagenase techniques, hepatocytes were prepared by dissociation of liver fragments obtained from hepatectomies performed for therapeutic purposes at the Service of Digestive Tract Surgery, Federal University of Triangulo Mineiro (UFTM). All patients gave free informed consent to participate and the study was approved by the Ethics Committee of UFTM.

Dissociation of human liver fragments by the EDTA method

Hepatocytes were prepared by dissociation of a liver fragment with EDTA. The perfusion solution had the following composition[7]: 140 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 1.6 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 2 mM EDTA, and 25 mM $NaHCO_3$. The EDTA solution was perfused through small catheters placed in the venous segments and fixed with 4.0 Prolene sutures at a constant temperature of 37ºC. A perfusion pump was used to guarantee continuous perfusion for 30 to 40 min at a constant flow rate of 3 to 4 ml/kg liver fragment/min. After perfusion, the liver fragment was placed in a 50-ml sterile glass container (Becker) filled with 20 ml HAM"S 12 culture medium at 4ºC and carefully cut until only small fragments were left. This suspension was then filtered through gauze bandage. The suspension obtained was ultracentrifuged twice at 5000 rpm for 2 min. The supernatant was aspirated and discarded and the cell mass obtained was weighed. Hepatocytes were separated on a pressure gradient by adding one volume of Percoll solution at a concentration of 1/10, using a proportion of 1 g cell mass for each 4 ml of Percoll solution. This solution was centrifuged at 6000 rpm for 5 min. Hepatocytes were counted in a Nageotte chamber. Cell viability was evaluated by determining membrane birefringence and by the 2% erythrosine exclusion test, and the results are expressed as percentage of viable cells.

Dissociation of human liver fragments by the collagenase technique

The method of *in situ* perfusion with collagenase described by Berry and Friend[8] and modified by Guilouzo[9] was used, which differs from the EDTA method in terms of the solutions employed and the time of perfusion. Perfusion solution – washing at a flow rate of 30 ml/min for 15 min: 160.8 mM NaCl, 3.15 mM KCl, 0.7 mM Na_2HPO_4 . $12H_2O$, 33 nM Hepes (N-[2-hydroxyethyl] piperazine-N-[2-ethanesulfonic acid]). Perfusion solution – dissociation at a flow rate of 15 ml/min for 20 min: 160.8 mM NaCl, 3.15 mM KCl, 0.7 mM Na_2HPO_4 . $12H_2O$, 33 mM Hepes, 0.075 mM $CaCl_2$, and 0.05% collagenase D.

Hepatocyte culture

The cells obtained with the two dissociation techniques were cultured in medium consisting of mixture of 75% Williams E (Gibco) and 25% Leiba 15 medium enriched with 100 mg/ml bovine serum albumin, 0.1 U/ml insulin, 30 IU/ml crystalline penicillin G, 100 mg/ml streptomycin, and 10% fetal bovine serum. The pH was adjusted to 7.4 with 2.2 mg/ml sodium carbonate. After 4 to 6 h of incubation at 37ºC in a humid atmosphere (95% air and 5% CO_2), the hepatocytes were found to be adhered to the bottom of the plate. The culture medium was then aspirated and replaced with the same fresh medium supplemented with hydrocortisone semisuccinate at a proportion of 4.5 x 10^5 mg/ml. This medium was changed daily until the end of the experiment. The hepatocytes obtained were cultured on 5-cm plastic Petri dishes in medium at a proportion of 1.5 x 10^5 cells/cm^2. Twenty-eight Petri dishes, including 14 for hepatocytes obtained by EDTA dissociation and 14 for hepatocytes obtained by the collagenase technique, were used.

Analysis of the preservation of albumin secretion by hepatocytes obtained with the EDTA and collagenase methods

The presence of human albumin was determined in the culture supernatant obtained daily from the 28 Petri dishes. Albumin was assayed in the aspirated culture medium using an antigen specific for the detection of human albumin, thus preventing contamination with the bovine albumin used in the preparation of the culture medium.

Results

Hepatocyte viability was compared daily in two plates from each group for a period of 7 days. The results showed that, during the first 4 days of the experiment, 70% of the cells presented birefringent membranes and were not stained with 2% erythrosine, and were therefore considered to be viable. During the first 3 days, hepatocyte viability was on average 71% in the EDTA group and 76% in the collagenase group, with no significant difference between groups. An expressive

reduction of cell viability was observed after the fifth day for both techniques, affecting the rates of viable cells. No significant difference was observed between groups at any time (Figure 1). The secretion of albumin by the cultured hepatocytes was preserved up to the seventh day. Mean albumin secretion during the first 3 days was 50 µg/ml in the two groups and a reduction of albumin production was observed from the fourth to the seventh day. Again, no significant difference was observed between groups at any time (Figure 2).

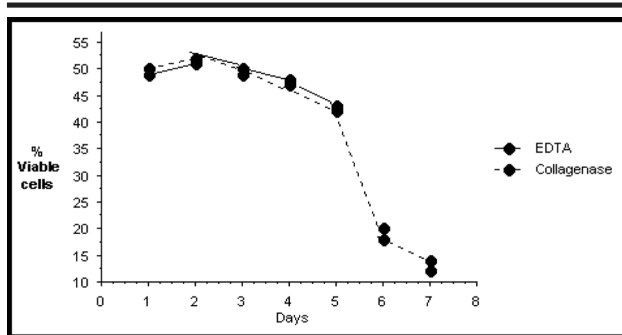

FIGURE 1 - Viability of isolated cultured hepatocytes obtained by EDTA and collagenase dissociation.

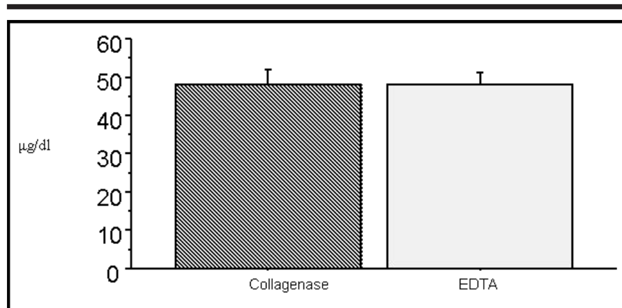

FIGURE 2 - Mean albumin secretion by the cultured hepatocytes (µg/dl)

Discussion

Liver transplantation has undergone great advances since 1963 when Starzl in the United States proposed homotransplantation of the liver in humans, a procedure which continues to be the only effective technique for functional replacement of the liver until today. Despite the advances in transplantation techniques, better immunosuppressive agents and improved care during postoperative recovery, 20 to 30% of patients with hepatic insufficiency continue to die while on the waiting list, mainly because of the small number of donors. In this respect, over the last few years, the development of a bioartificial liver or of viable hepatic cell transplants has raised the interest of several research centers in an attempt to temporarily replace the failing liver, maintaining the patient alive until a compatible donor is found, or even to replace treat isolated metabolic disorders [10,11]. To obtain isolated cells with preserved functions, it is necessary to dissociate the hepatocytes from the liver to separate them from biliary, vascular and connective tissues which are often responsible for rejection of the organ. There are still no standardized liver dissociation techniques; however, all techniques propose the perfusion of solutions through the portal vein [5] in intact livers or small liver fragments [11,12], with the methods differing in terms of the solution used for perfusion witch EDTA [7], hyaluronidase, collagenase or a combination of these solutions[13]. In the present study, cell viability was 85% and 83% after dissociation with EDTA and collagenase, respectively, rates higher than those reported by other investigators [14]. Up to the third day, mean viability was found to be similar for the two techniques and a marked quantity of viable hepatocytes was observed, which might be used in hemodialyzers or in studies on new drugs or cellular genetic modifications [2,9,15]. The present results were similar to those reported in previous studies employing *in situ* dissociation of rat livers. Cell viability was excellent and no difference was observed between dissociation with EDTA and collagenase. We therefore recommend the use of EDTA as a dissociation solution because of its much lower cost compared to collagenase. Another advantage of EDTA is that its action is interrupted at the end of dissociation, in contrast to collagenase, thus preserving cellular functions.

Conclusion

Based on the present experiment we conclude that cell viability and preservation of albumin secretion by hepatocytes are similar for the EDTA and collagenase techniques.

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Conflict of interest: none
Financial source: Research Foundation of Minas Gerais

Received: August 15, 2006
Review: September 12, 2006
Accepted: October 10, 2006

How to cite this article:
Crema E, Werneck AM, Martins JR A, Ribeiro LBP, Lima TS, Silva AA. Comparative analysis of preparation of human hepatocytes by the ethylenediamine tetraacetic acid and collagenase technique. Acta Cir Bras. [serial on the Internet] 2007 Jan-Feb;22(1). Available from URL: http://www.scielo.br/acb.