Improvement of the Original Isolation Procedure for Hormone Studies in Short-Time Culture

Mukadder Atmaca

SUMMARY

Earlier studies indicated that hormone responsiveness of cells and metabolic activity was lost during various of experimental procedure. In the light of this observation, I aimed to investigate to obtain optimal conditions for short time cultured hepatocytes and also to determine the type of test can be used to evaluate suitablity of hepatocytes for hormones studies. During the isolation period 50 IU/ml and 100 IU/ml collagenase were used. Adrenaline (10^{-6}M) was used to measure sensitivity of hepatocytes to hormones and glycogenolsis was measured at the end of 2hr incubation period. Adrenaline significantly increased gylcogenolysis (Control: 0.16±0.01 mg/2hr; Adrenaline: 0.30±0.01 mg/2hr) only when the 50 IU/ml collagenase was used and the viability of the cells were over 95%.

Viability tests were applied to hepatocytes that obtained by using 50 IU collagenase. Cellular glutathione, methylthiazoltetrazolium reduction, lactatedehdrogenase leakage, ATP level measured to determine viability following the attachment and incubation period. No differences were observed at the end of each period.

Altogether, the present study indicated that membrane integrity and metabolic function of the hepatocytes can be improved by modifying slightly the original procedure of Reese and Byard.

Key Words: *Hormon, Cell Viability, Metabolic Activity*

Kısa Süreli Kültür Ortamında Hormon Çalışmaları İçin Özgün İzolasyon Prosedürünün İyileştirilmesi

ÖZET

Daha önceki çalışmalar izolasyon prosedürleri esnasında hücrelerin hormona yanıtının kaybolduğunu ve metabolik aktivitenin azaldığını göstermiştir. Bu nedenle, çalışmamda kısa süreli izole karaciğer hücre (hepatosit) kültürleri için optimal koşulların sağlanması ve hormon çalışmalarında daha uygun bir yöntem geliştirmeyi amaçladım. Hücre izolasyonu için 50 IU/ml ve 100 IU/ml kollejenaz kullanıldı. Sıçan hepatositlerin hormonlara duyarlılığının ölçümünde adrenalin kullanıldı ve glukoz çıkışı (glikojenoliz) 2 saatlik inkubasyon peryodu sonunda ölçüldü. Adrenalin'nin (10^{-6}M) glikojenolize etkisi yanlızca 50 IU/ml kollojenaz kullanıldığında ve viabilitenin %95'in üzerinde olduğu durumlarda anlamlı görüldü (Kontrol: 0.160±0.01 mg/2saat; Adrenalin: 0.30±0.01 mg/2saat).

Düşük konsantrasyonda kollejenazla izole edilen(50IU/ml) ve canlılığı %95 üzerinde olan hepatositlerin, mikroplatlere yapıştığı süreç sonunda (1saat) vede bunu takip eden inkübasyon sürecinin sonunda, glutatyon içeriği, metiltyotetrazolum redüksiyonu, laktatdihidrojenaz çıkışı, adenozindifosfat düzeyi ölçüldü. Bu süreçler sonunda belirtilen ölçümlerde değişiklik gözlenmedi.

Sonuç olarak, bulgularımız Reese and Byard'a ait özgün izolasyon prosedürünün modifiye edilmesiyle hepatositlerin metabolik faaliyetlerinin ve membran bütünlüğü'nün korunabileceğini göstermektedir.

Anahtar Kelimeler: *Hormon, Hücre Canlılığı, Metabolik Aktivite*

University of Dicle, School of Medicine, Department of Physiology

INTRODUCTION

A wide range of experimental preparations have been used to study effect of hormones on liver metabolim. These include, the intact animal, the whole perfused liver, liver slices and cubes, liver homogenates and isolated hepatocytes. Each of the techniques have certain benefits and limitations. Among this techniques use of isolated hepatocytes has the advantage that the researchers can control the extracellular environment, allowing one to study the action of a single hormone in the absence of other confounding variables.

A successful preparation of isolated hepatocytes must retain the functional properties of these cells as observed in the intact liver. Therefore, it is essential to assess cellular damage and metabolic integrity during the experimental period. Such measurements will reveal whether or not preparation has metabolic characteristics similar to those observed in vivo. A number of assays are currently being used to assess the reproducibility of the cell isolation procedure. The permeability of cell membrane during cell preparation, trypan blue exclusion, and throughout the incubation period lactate dehydrogenase (LDH) leakage and cellular glutathione (GSH) level are used to assess cell viability. In addition methylthiazoltetrazolium (MTT) reduction, maintenance of the cellular adenosine diphosphate (ATP) concentration, GSH content are all measures function (1,2,3).

Measurement of membrane integrity alone provides no guarantee that the cells are metabolically viable even if the membrane is still intact and so these measurements may provide inadequate information regarding subtle changes during short incubation periods. It is therefore essantial to measure any metabolic activity that relevant receptor activities, such as glycogenolysis. In this study, hepatocytes were isolated by perfusion of liver lobes with collagenase, essentially as described by Reese and Byard (4). The morphological appearance of hepatocytes under the light microscope, permeability of the cell plasma membrane during cell preparation (trpan blue exclusion) and throught the incubation period (LDH leakage) are used to assess cell viability in the present study. In addition MTT reduction, maintenance of the cellular ATP concentration, GSH content and glycogenolysis are used to detect cellular functions of hepatocytes.

MATERIALS and METHODS

Adult male Wistar rats (150-180g) were obtained from the University of Nottingham Biomedical Services Unit. They were housed at a constant room temperature of 22°C and had free access to standard laboratory diet and tap water. Culture medium, adrenaline, Trinder glucose assay kit, collagen and collagenase ATP monitoring reagent, MTT, LDH were purchased from Sigma Chemicals Co. (Poole, UK).

Hepatocyte isolation procedure

Rats were killed by cervical dislocation. Liver lobes removed as quick as possible and rinsed with Hanks buffer. The lobs cannulated and then perfused with the buffer for a few minutes to flush out the blood. After allowing the lobes to blanch, the buffer containing 0.5M EGTA was perfused for 15 min. to allow the removal of Ca^{++} from the liver lobes. Following this period, the lobes were perfused with recirculating Hanks buffer,pH 7.4, containing collagenase (Sigma type IV; 100 IU/ml or 50 IU/ml) and 5ml $CaCl_2$ for 15 min until the lobes were soft and starting to disintegrate. All solutions were gased with 95%O_2 and 5%CO_2. At the end of this stage lobes were placed in a few ml of Hanks Buffer and minced gently with a pair of scissors. The resulting suspension was passed through a tea strainer and then centrifuged at 600rpm for a few seconds. The suspension was removed and the pellet resuspended in the buffer. This procedure was repeated for second time for 10 min. After the second spin, cells were resuspended in Leibovitz medium (L-15 medium) including newborn calf serum and centrifuged (50g for 10 min) in a 90% percoll solution to improve the separation of viable and nonviable cells, as described by Kreamer et al. (5).

Viability of cell suspension was determined using the trypan blue dye exclusion test. A sample of hepatocytes suspension (0.25ml) was mixed with trypan blue (0.1ml; 0.4% dye solution). A Neubauer haemmocytometer was used to count the number of viable and non-viable cells and the percentage of viability was calculated. Suspensions with a viability~80% and over 95% were used for hormonal response in this study.

Hepatocytes were suspended in (L-15) medium containing 10% calf serum and dispensed into collagen-coated six-well plates at a density of $1x10^6$ cells per well. Following one hour attachment L-15 medium removed from the plates and proper chemicals were added to the dishes for cellular GSH, ATP level, LDH leakage and MTT reduction assay. Alternatively L-15 medium was replaced with 2 ml of Hanks buffer pH: 7.4 including 10^{-6}M concentration of adrenaline or the cells were incubated for 2 hr within Hanks buffer without adrenaline. After the 2 hr incubation period, 100µl of medium was assayed for glucose according to the instruction in the assay kit. Proper solutions were added to the emptied plates for measuring cellular of GSH, ATP, LDH leakage and MTT reduction assay.

MTT reduction was determined by modification of method of Denizot and Lang (6). The method of measurement of glucose release by glycogenolysis was based on the colorimetric reaction described by Trinder (7) ATP analysis was performed using the LKB Luminometer which detects light produced by luciferase using luciferin and ATP as a substrates. LDH levels was measured as described by Hammon et al (8). Cellular GSH content was measured by the assay of Saville (9)

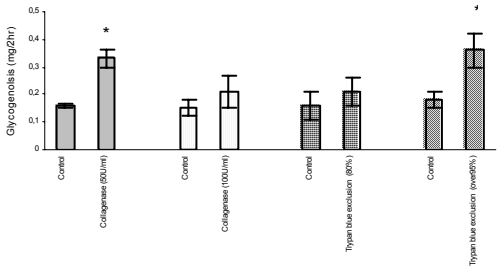
Data are presented as mean ± SEM of at four separate experiments, each value representing the mean of a minimum of three measurements. Statistical analysis was undertaken using unpaired t-tests or analysis of variance (ANOVA)/ Dunnett's test as appropriate. A value of $P<0.05$ was considered significant.

RESULTS

By the end of the preparative procedure, the features of hepatocytes were examined under a light microscope. They were having a reasonably homogeneous cell population and a typical cell feature.

In earlier studies of hepatocytes hormone-responsiveness, some evidence for lose of hormone receptors was reported (10). Indeed, in my initial study no glycogenolytic response (which is receptors mediated event) was observed to adrenaline (Figure 1) when high concentrations of collogenase were employed which suggested concentration for metabolic studies (100 IUnit/ml). When the concentration of collagenase used in this study reduced to half (50 IUnits/ml) of the initial concentration (Figure 1). Hormone responsiveness of the cell to hormones was observed when exposure time of the hepatocytes to collagenase during perfusion was reduced (Figure 1).

Figure 1. Effect of different concentrations of collagenase and trypan blue exclusion on glycogenolysis in the presence of adrenaline (**10^{-6}**M)



Reults are mean ± SEM of 4 separate experiments. * Where indicated, values are significantly different from control at $*P<0.5$

The receptor mediated activity of adrenaline was only observed in cell preparations in which 95% or more of the cells were intact and excluded trypan blue (Figure 1)

Following slight modification related to the collagenase I decided to see whether this modification could help to maintain metabolic activity of the hepatocytes. Plasma membrane integrity of hepatocytes were assessed by cellular LDH level following the attachment period (1hr) and incubation period (2hr). No

significant differences was observed at LDH level at the end of the each period (Table 1).

Table 1. Viability of hepatocytes throughout the incubation period (3hr)

	Conditions	
	Attachment Period (1hr)	**İncubation Period (2hr)**
GSH Level (nmol/well)	36.04± 1.55	33.01± 1.01
LDH leakage (µmolpyruvate /ml/min)	1.28±0.10	1.21± 0.10
MTT reduction (Absorbance)	0.16±0.01	0.16 ± 0.01
ATP level (nmol/well)	1.3± 0.1	1.4± 0.2

Values are mean±SEM of 4 experiments.

Measurement of ATP level gives some idea of the changes in cellular energy levels. Therefore, cellular ATP level was examine at each stage of the experiment. The cellular ATP level was remained the same (Table 1). Determination of cellular MTT reduction is another assay that can be used to measure cellular integrity. Similar the two assays, no differences was observed the two different time point of the incubation (Table 1). The cellular GSH level decreased to the the end of the incubation period but this was not statistically significant (Table 1).

DISCUSSION

Conventionally, different number of techniques have been used in the study of liver metabolism. Each one has advantage and disadvantage over the others techniques. Cultured hepatocytes is the more commonly used system among the others and can applied for long and short term. Advantages of cultered cells as opposed to the other techniques is that one animal may provide sufficient cells to test the effects of several chemicals, and that animal used to set up cultures may act it is own control. Earlier studies indicated that hormone responsiveness of cells was lost during the various of experimental procedure. Therefore, slight modifications of original isolation procedure was took place to improve hormone studies.

It has been well established that damaged cells have lost the ability to maintain and provide energy for metabolic function (11). A successful preparation of isolated hepatocytes must retain functional properties immediately after the isolation procedure and during the incubation period. Therefore, to determine the quality of the hepatocytes, a series of selected assays were used to determine the membrane integrity and metabolic function.

Previous studies indicated that hormone responsiveness of hepatocytes was lost during the isolation procedure and this was associated with action of collagenase during the perfusion of the liver (10). In my initial studies no effect of adrenaline on glycogenolysis were observed when 100 IU/ml of collagenase, which is the suggested concentration in general used for disintegration of the cells during the isolation period. When the collagenase concentration was reduced to half of the original concentration, 50 IU/ml, effect of adrenaline on glycogenolysis were observed. This modification also helped to enhance cell viability from 80 to 95%, when cell viability was low the hepatocytes did not respond to the adrenaline. The results of this study were in agreament with earlier studies of Garrison and Haynes (10). At each stage of the incubation period, possible morphological differences of hepatocytes must be controlled. Therefore, the feature of the hepatocytes were examined under light microscope at end of the each period. The undamaged hepatocytes were generally oval or spherical in shape, whereas the irreversibly damaged cells rapidly took up the trypan blue and so, contained blue nuclei.

Damaged cells lost the ability to maintain and provide energy for metabolic function and growth. It has been indicated (11) that under hypoxic condition or at low tempratures maintenance of Na^+ and K^+ gradients in hepatocytes is abolished because of disturbance in the energy-dependent transport mechanism. Therefore, particular attention must be paid to avoid hypoxia and maintain good temperature control during perfusion of the liver.

Viability tests were applied to hepatocytes that obtained by using 50 IU collagenase

during the isolation procedure and the cells that have over 95% of trypan blue exclusion. Cellular GSH content, MTT reduction, LDH leakage and ATP level.

GSH plays a fundamental role in normal cellular physiology. Previously, it was indicated that GSH content of hepatocytes decreased over experimental period (12). The results presented here support this finding. Cellular GSH level decreased during the incubation period, but not found statistically significant. It is possible that the fall in GSH was related to use of GSH in attachment and incubation period as mentioned by Hammond and Fry (12). The avability of these methods provides a powerful set of tools for probing the contribution of GSH to a number of cellular proccess such as toxicity, and the effects of endogenous agents on GSH metabolism.

MTT reduction assay is commonly utilised to estimate cellular viability (1) and measures tetrazolium conversion to colored formazan product. It is suggestable for deteremination of viability among the others assays because of its simplicity, high speed, precision, reproductibility and and low cost. Futhermore, MTT reduction is widely used in biochemistry as a detection metod of a variety of mitochondrial dehydrogenase activity (13).

Mesurement of the ATP level is the another useful indicator of cell function. Assessment of ATP level gives some idea of the changes in cellular energy levels. It is claimed that maintenance of cellular ATP is a good prognostic indicator for success of hepatic transplantation (14, 15).

It has been well established that damaged cells have lost the ability to maintain and provide energy for metabolic function and growth. Therefore to test membrane integrity of the hepatocytes, I also examined cellular ATP level. No differences were observed at the end of isolation and incubation period. The results of this study shows satisfactory metabolic competence which is in agrement with Page et al (2).

Measurement of membrane integrity alone provides no guarantee that cells are metabolically viable even if the membrane is still intact and so these measurements may provide inadequate information regarding subtle changes during short incubation period. LDH leakage was the last assay in present study to confirm that following slight modification the viability of the hepatocytes remained the same. Cellular LDH leakage was measured at the end of attachment and incubation period. No substantial difference was observed, like the other viability assays.

Altogether, the present study provides evidence for the validity of using short term monolayer cultures to look at the effects of hormones and metabolic studies by slightly modifying isolation procedure for short term culture.

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Corresponding Author:

Mukadder ATMACA
University of Dicle, School of Medicine,
Department of Physiology, Diyarbakır
E-mail: heja@dicle.edu.tr