ปีที่ ๑๘ เล่มที่ ๑
มกราคม - มิถุนายน ๒๕๒๙

Vol. 18, No. 1
January - June 1986

วารสารสำนักงานคณะกรรมการวิจัยแห่งชาติ
Journal of the National Research Council

ISOLATED LIVER CELL PREPARATIONS*

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ABSTRACT

Suspension of hepatocytes was isolated from the livers of male Wistar rats weighing 200-250 g by using the method of Berry and Friend as modified by Stacey and Priestly. Morphological features, plasma membrane integrity and metabolic capability were used as the criterion for cell viability. The successful method to obtain good cell preparations must involve three critical steps; namely, exposure of the liver to a calcium free medium, digestion with collagenase in a recirculating system and gentle mechanical treatment. Albumin (1.2%) in the final medium which was used for suspending cells is necessary to maintain cell viability. Cell yields of 2-3 $\times$ 10^8 cells were obtained with a trypan blue exclusion index of 90-98%. Cell preparations with a trypan blue exclusion index of less than 90% were not recommended for any studies.

บทคัดย่อ

เซลล์ตับอิสระเตรียมได้จากการแยกเซลล์ออกจากตับของหนูขาวใหญ่ เพศผู้ สกุล Wistar น้ำหนัก 200–250 ก. โดยใช้วิธีของ Berry และ Friend ซึ่งปรับปรุงโดย Stacey และ Priestly ลักษณะรูปร่างของเซลล์ ความคงตัวของผนังเซลล์ และความสามารถของเซลล์ในการเมตาบอลิสมใช้ในการตรวจสอบว่า เซลล์ที่เตรียมได้นั้นเป็นเซลล์ที่มีชีวิตอยู่หรือไม่ วิธีการเตรียมเซลล์ที่ได้ผลดีจะต้องเกี่ยวข้องกับขั้นตอนสำคัญ 3 ขั้นตอน

*This study was supported by Chulalongkorn University Research Fund 1982-1983.

คือ การผ่านน้ำยาที่ไม่มี Ca^{+2} เข้าไปในตับ การย่อยส่วนที่ติดกันของเซลล์ออกด้วยเอ็นไซม์ collagenase ในระบบหมุนเวียนกลับ (recirculating system) และการแยกเซลล์ออกจากกันด้วยแรงเบา ๆ พบว่าการเติม albumin (1.2%) ลงไปในน้ำยาสุดท้ายมีความสำคัญต่อการมีชีวิตอยู่ของเซลล์ เซลล์ตับอิสระที่เตรียมได้ในแต่ละครั้งจะมีค่าประมาณ $2-3 \times 10^8$ เซลล์ โดยมีค่า trypan blue exclusion index อยู่ระหว่าง 90–98% สำหรับเซลล์ที่มีค่า trypan blue exclusion index ต่ำกว่า 90% นั้นไม่เหมาะที่จะนำมาใช้ในการศึกษาใด ๆ

INTRODUCTION

Early attempts to isolate hepatocytes by employing mechanical force[3] and subsequently, perfusion of the liver with Ca^{+2} or K^{+} chelators were unsuccessful in obtaining viable cells with high yields.[9,12] The major development was the introduction of collagenase and hyaluronidase for isolation of rat hepatocytes. This technique was first described by Howard et al.[6,7] The method was further modified by several investigators. Berry and Friend[2] introduced a recirculating perfusion technique, whereas Wagle and Ingebretsen[22] simplified the procedure by using collagenase as the only digestive enzyme. Seglen[16] preperfused the liver with calcium-free medium prior to the perfusion with collagenase in a calcium containing medium. These modifications decreased the perfusion time and increased viable cell yields.

The viability of cells was the important condition for relevant results. Therefore, several criteria for judging the intactness of hepatocytes were followed. These included the morphological features,[9] plasma membrane integrity,[1,17] cell respiration[12] and the capability to maintain biosynthetic and catabolic reactions.[1,9]

The factors involved in selecting suitable viability tests were the simplicity and sensitivity of the methods. In this study, the trypan blue exclusion test was used routinely to establish the viability of freshly prepared hepatocytes, while K^{+} retention and alanine aminotransferase (ALT) leakage were used to monitor the plasma membrane integrity during the course of an experiment.

The overall objective of this study was to set up a system for isolating rat liver cells. The criteria used for cell viability were based upon plasma membrane integrity and metabolic capability. Any preparations which were satisfied by these criteria should be recommended as a suitable model for studies on liver function, drug metabolism and drug induced toxicity.

MATERIALS AND METHODS

Male Wistar rats weighing 200-250 g from Mahidol University Central Animal House were used as liver donors. They were allowed free access to food (Charlick's M and V mouse cubes) and water. Some experiments involved food deprivation (24 h fasting). Surgery was performed between 8.30-9.30 a.m. for each experiment. Under ether anesthesia, hepatocytes were isolated by the method of Berry and Friend[2] with some modifications by Stacey and Priestly.[18] Liver was perfused with Ca^{+2} free physiological solution (96 mM NaCl, 1.4 mM

KCl, 0.7 mM $MgSO_4$, 2.5 mM KH_2PO_4, 30 mM $NaHCO_3$ and 21.7 mM sodium glüconate, equilibrated with 95% O_2/5% CO_2 at pH 7.4) via the portal vein. When the perfusion of all hepatic lobes was rapid and complete, the liver was perfused with 100 ml of 0.05% (w/v) collagenase (sigma type IV) in the same calcium free buffer equilibrated with carbogen (95% O_2/5% CO_2), under the recirculating condition. Flow maintained at 30-35ml/min with a pressure head of 20 cm of water, and temperature was maintained at 37°C throughout the procedure.

After perfusion with collagenase (10-15 min), the liver was dispersed with a blunt spatula in 50 ml of fresh collagenase buffer (0.05%) and incubated (37°C, 80 oscillations/min) for 10 min. Bovine serum albumin (BSA) was added to give a final concentration of 12 mg/ml and cells were harvested through nylon mesh (250 - 61 μm). Hepatocytes were separated from other cells and cellular debris by differential centrifugation (50 G, 1 min). The resulting cell pellet was washed twice with this fresh physiological medium containing 12 mg/ml BSA and once with the incubation medium.[10] The final cell pellet was resuspended in the incubation medium at the concentration of approximately 4-5 $\times$ 10^6 cells/ml. Viability of freshly isolated hepatocytes was determined routinely by the trypan blue exclusion test.[17] Cell preparations with a trypan blue exclusion index of less than 90% were never used in any studies.

Hepatocyte suspensions (3 ml) were incubated for 30 min at 37°C. Aliquots (0.5 ml) of cell suspension were centrifuged at 50 G for 1 min. ALT activity in the supernatant was determined by the method of Reitman and Frankel.[14] The cell pellet was extracted with 1 ml 3% (w/v) perchloric acid (PAC), then centrifuged at 3,500-5,000 rpm for 5-10 min. The resulting supernatant was diluted with distilled water and the K^+ concentration determined by flame photometer.

The gluconeogenic capacity of isolated cells was evaluated using the method of Cornell and Filkins.[4] Rats were fasted 24 h prior to isolation. After 1 h of incubation with 10 mM lactate, the samples were deproteinized with 1 ml each of 1.8% barium hydroxide and 2.0% zinc sulfate and centrifuged at 1,000 G, then 1 ml samples of supernatant were analyzed for glucose using the o-toluidine method.

Aminopyrine demethylase activity in cell suspension was determined by the method of Mazel.[11] Aliquots (2.5 ml) of cell suspension were incubated with 1 mM aminopyrine for 1 h. Formaldehyde formed during the incubation was trapped as the semicarbazone (by semicarbazide in the incubation medium) and measured by the colorimetric procedure of Nash.[13]

Cell counting, cell appearance and trypan blue exclusion tests of isolated hepatocytes were performed using a Nikon Optiphot light microscope at 40 $\times$ and 100 $\times$ magnification.

Electron micrographs of isolated cells were done by the transmission electron microscope. Cells were fixed with 1-2% OsO_4 in 0.1 M phosphate buffer, pH 7.3 for 2 h. After washing with phosphate buffer, cells were dehydrated with ethanol (from 35% up to 100%) then infiltrated with propylene oxide and plastic mixture for 2-3 days. The embeded cells were sectioned with ultramicrotome and dyed with uranyl acetate and lead citrate prior to photomicrography.

RESULTS

The morphology of freshly isolated adult rat hepatocytes was shown in Figure 1. Only viable cells with intact membrane excluded trypan blue. Cells obtained by this technique usually had the trypan blue exclusion index of 90-98%. They retained a rounded appearance and there was an abundance of normal-appearing intracellular organelles as shown in the electron micrographs (Figure 2).

The analysis of cell membrane integrity by K^+ retention and ALT release before and after 30 min incubation was shown in Table 1. Values represent the mean ± S.E. of 5 separated experiments.

Gluconeogenesis from lactate and microsomal aminopyrine demethylase activity were used as indices for metabolic capability of cells. Values as mean ± S.E. from 3-5 separated experiments were shown in Table 2.

Trypan blue exclusion index was performed routinely for cell viability test. Cell preparations with a trypan blue exclusion index of less than 90% were never used in any studies. Addition of bovine serum albumin (1.2%) to the final medium for suspending cells improved cell viability as detected by trypan blue exclusion test (Table 3).

DISCUSSION AND CONCLUSION

It was necessary to verify that isolated hepatocytes prepared by this technique were representatives of normal liver cells *in vivo*. The criteria used for cell viability are morphological features, plasma membrane integrity and capacity of cells to maintain biosynthetic and metabolic reactions.

For morphological features, nearly all of isolated cells retained their normal spherical shape with normal distribution and structure of organelles (Figures 1,2). The electron micrographs (Figure 2) of these cells were compared favourable with those reported by other investigators.[2,8,9,15,22] This suggested that cells isolated by the described procedure did not alter the morphology of cells.

Plasma membrane integrity were measured using trypan blue exclusion test, K^+ retention and ALT release. The trypan blue exclusion test was used routinely to establish the viability of freshly isolated cells, while K^+ retention and ALT release were used to monitor the plasma membrane integrity during the course of an experiment. The average values of initial K^+ content and ALT release (Table 1) were consistent with those reported by Gottschall et al.[5] and other investigators.[18,19,20] In Table 1 the values of K^+ retention and ALT release were persisted after 30 min of incubation. This indicated that the isolated cells could maintain

their intact membrane and plasma membrane integrity before or after incubation in the control environment.

The metabolic capability of cells as measured by gluconeogenesis from lactate and microsomal aminopyrine demethylase activity (Table 2) was comparable to other cell preparations prepared by other investigators.[4,16,21]

Suitable medium was required for good cell viability. Bovine serum albumin (1.2%) used for suspending cells in the final medium was found to be necessary (Table 3). Different laboratories seemed to use different kinds of medium, depending on their needs in experiments.

In conclusion, isolated hepatocytes obtained by this technique were morphologically intact and metabolically active. So they could be used as the representatives of normal liver cells *in vivo*.

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Table 1. Analysis of cell membrane integrity.

Incubation time (min)	K^+ (μM/g cells)	ALT (IU/g cells)
0	53.30 ± 1.11	3222.22 ± 104.17
30	52.93 ± 1.35	3553.82 ± 121.50

Values shown are mean ± S.E. (n = 5)

Table 2. Matabolic capability of cells.

Gluconeogenesis from lactate (uM Glucose formed/g cells/h)	Microsomal Aminopyrine Demethylase Activity (uM HCHO formed/g cells/30 min)
mean ± S.E. 21.00 ± 0.83 No. of experiment (n) = 5	mean ± S.E. 1.19 ± 0.11 No. of experiment (n) = 3

Table 3. Effects of bovine serum albumin (BSA) on cell viability.

Trypan blue exclusion (%)	
Without BSA	With BSA
mean ± S.E. 73.8 ± 1.2 n = 5	mean ± S.E. 94.8 ± 0.9* n = 5

*Significantly different from control (without BSA) ($P < 0.05$, t-test)

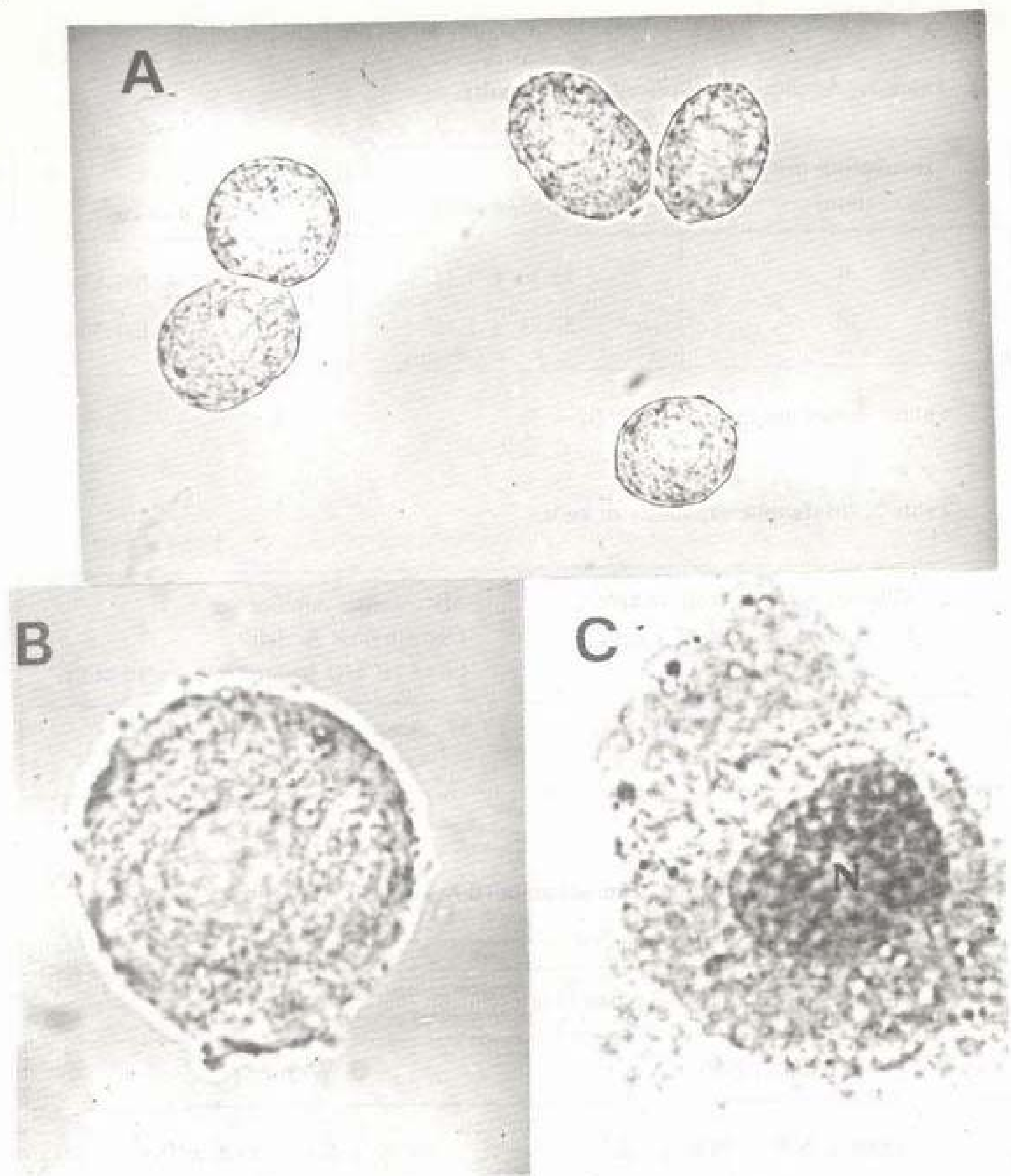

Fig. 1 A. Suspension of freshly isolated rat hepatocytes in modified Krebs-Henseleit physiological solution (× 40).
B. Freshly isolated rat hepatocytes with rounded appearance (× 100).
C. Dead cell with stained nucleus (trypan blue) (× 100).

N = Nucleus

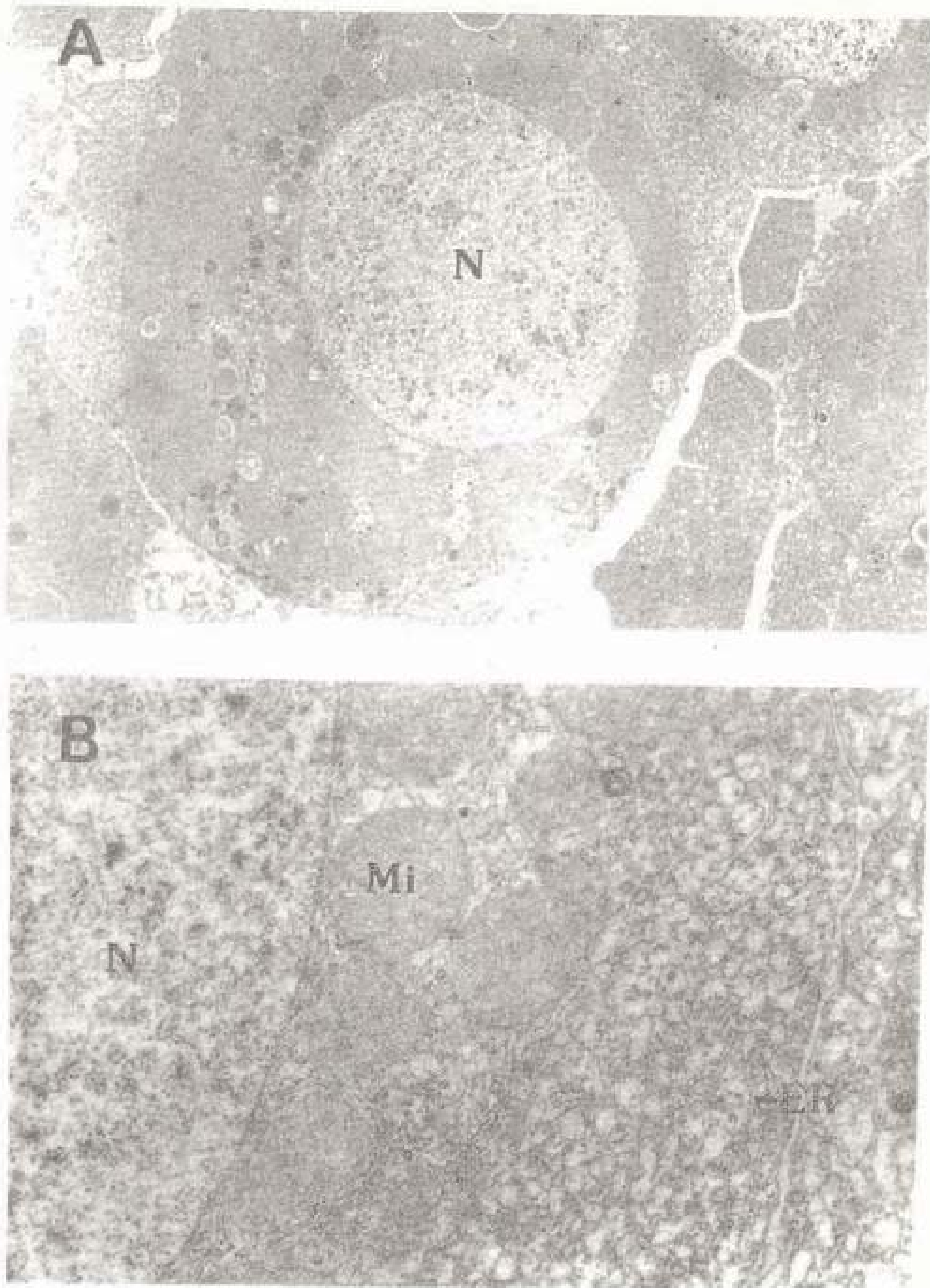

Fig. 2 A. Electron micrographs of freshly isolated rat hepatocytes with normal cell structure and organelles (× 3,000).

B. Electron micrographs of isolated rat hepatocytes with normal distribution and structure of mitochondria and endoplasmic reticulum (× 15,000).

N = Nucleus Mi = Mitochondria

ER = Endoplasmic reticulum