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# Analysis and Optimization of Nutritional Set-up for Murine Pancreatic Acinar Cells

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## ABSTRACT

**Context** Pancreatic acinar cell cultivation poses a serious problem due to limitations in the *in vitro* survival time despite variations of dissociation protocols, culture media and nutrient supplements.

**Objective** To establish a long term culture of murine pancreatic acinar cells which retain their viability, monolayer formation and responsiveness to secretagogues. In order to investigate the mechanism of the short-life of acinar cells studied *in vitro*, we studied their survival under the influence of different supplements on nutrient media.

**Interventions** Dissociated pancreatic acini were prepared from BALB/c mice pancreata by collagenase digestion supplemented with bovine serum albumin fraction V and soybean trypsin inhibitor. A nutrient set-up was designed for their long term survival *in vitro*.

**Results** It was observed that mouse pancreatic acinar cells dissociated in presence of bovine serum albumin fraction V and soybean trypsin inhibitor result in 95% viability. Further cultivation of these acinar cells in Waymouth's MB 752/1 medium supplemented with 10% fetal calf serum (v/v), soybean trypsin inhibitor, bovine serum albumin, dexamethasone, and epidermal growth factor results in their survival for more than 6 days in culture with 85% viability, retention of the secretagogue responsiveness and formation of a monolayer without any extracellular matrix coating.

**Conclusions** Our study clearly demonstrates that the addition of soybean trypsin inhibitor to culture medium reduces zymogen granule fragility and acinar cell death, thus increasing their viability for sufficiently long periods. The present study offers an excellent, *in vitro* model for the investigation of exocrine dysfunction in response to acinar cell injury.

## INTRODUCTION

The complex physiology of the pancreatic acinar cell and its relative inaccessibility *in vivo* has warranted an effective *in vitro* model of the pancreas which has been pursued for many years. Multiple methods of acinar cell and dispersed acini isolation have been developed, but each one has its inherent limitations [1]. One of the widely accepted techniques of preparing isolated pancreatic acinar cells for *in vitro* studies is to subject the excised pancreas of an animal (guinea pig, rat or mouse) to collagenase digestion [2, 3]. The tissues are exposed to chelating agents and mechanical shearing is achieved by vigorous pipetting. Unfortunately, these cells are abnormal structurally and functionally. They demonstrate loss of microvilli and normal apical structures; they are poorly responsive to secretagogues, and they are viable for a few hours. Moreover, many essential cell surface receptors are damaged during the digestive process. Another commonly used model that allows the *in vitro* study of acinar cells is based on the isolation of intact acini as opposed to individual cells [4]. The main aim of this method was multifolds; it involved maintaining of normal

acinar cell-to-cell communication and decreasing the caustic treatment of the cell membranes, which are vital to many cellular functions. The dispersed acini model eliminates the chelation process used to isolate individual acinar cells and employs a gentler means of mechanical shearing. The advantages of dispersed acini are better maintenance of a more normal intracellular and intercellular architecture, improved responsiveness to secretagogues and modestly extended, albeit limited, viability.

Over the last few years, several methods have been described for tissue culture of pancreatic acinar cells which produce cultured cells having vastly different characteristics [1, 4, 5, 6, 7]. Despite variation in the dissociation protocols, culture medium and nutrient supplements it has not been possible to maintain primary cultures of pancreatic acinar cells for more than 4 days [8]. Why these culture systems differ remains unclear; the variations in the culture systems observed could be attributed to the differences in the species from which the cells are cultured, the techniques by which the cells are isolated, the morphological form of the cultured cells (acinar cells versus acini) and the composition of the media. It has been seen that rat acinar cells cultured for 10 days in serum-containing medium exhibit a normal morphology, but do not grow in culture and are not responsive to hormones containing medium [5]. It has also been reported that rat acinar cells cultured in serum containing medium on reconstituted extracellular matrix form aggregates of acinar cells on the matrix showing an apparent acinar-like structure [9], while in another study monolayer formation and growth of cells in the culture within 2 days [10] has been reported. Logsdon and colleagues have cultured murine acini short-term [6] and acinar cells long term [11].

Recent studies have demonstrated that zymogens may be activated within the pancreatic acinar cells [12, 13]. The systemic effects of these active pancreatic enzymes result in widespread tissue damage. Agents that efficiently inhibit intracellular zymogen activation and block the activity of released enzymes may prevent acinar cell damage. We have previously reported that high or low pH, makes the zymogen granules fragile resulting in lysis of the cells [14]. Historically, the unique properties of albumin have permitted improved tissue protection in numerous models of solid organ preservation [15, 16]. Since albumin is known to protect tissue morphology during *in vitro* culture we have used bovine serum albumin fraction V (BSA) in the digestive medium. It has been reported that if the digestion is carried out in a solution containing 10% BSA, the release and activation of neutral protease activity, which occurs during collagenase digestion of rat, porcine, canine, and human pancreata, can be dramatically reduced [17]. Moreover, albumin possesses the capacity to act as a scavenger of oxygen free radicals [18]. We have therefore made an attempt to define the necessary requirements for *in vitro* culture of pancreatic acinar cells and we have examined the effect of different dissociation conditions, media and additives on survival of primary culture of murine pancreatic acinar cells.

## MATERIALS AND METHODS

### Animals

Six-8 week-old BALB/c mice of both sexes were used for this study. All animals were obtained from an inbred population of BALB/c mice maintained at the animal facility of the National Centre for Cell Science, Pune, India. Pancreatic tissue was aseptically removed from a group of two to four mice which were killed by means of $CO_2$ asphyxiation.

### Acinar Cell Isolation

Dissociated pancreatic acini were prepared from BALB/c mice pancreata by collagenase digestion following a slightly modifications Oliver's procedure [5]. Briefly, animals fasted overnight and pancreatic tissue was dissected free of mesenteric fat, minced, washed with phosphate buffered saline (pH 7.2) with 0.01% soybean trypsin inhibitor (Sigma

Chemical Co., St. Louis, MO) and incubated with 20 mL of 0.02% trypsin and 0.25% EDTA for 5 minutes at 37 °C while shaking 120 to 140 cycles per min. After centrifugation at 500 *g* for 2 min, the tissue was rinsed with Waymouth's MB 752/1 medium (Sigma Chemical Co. St. Louis, MO, USA) with antibiotics (penicillin-streptomycin) and 0.1 mg/mL soybean trypsin inhibitor, 5 mg/mL bovine serum albumin fraction V (Sigma Chemical Co., St. Louis, MO) and 20% fetal calf serum. The tissue was centrifuged at 500 *g* for 2 min, and the pellet was resuspended in 20 mL of digestive solution containing 1 mg/mL collagenase P (Bohringer, Mannheim, Germany), bovine serum albumin fraction V 0.2 mg/mL and digested for 15 min at 37 °C while shaking at 120-140 cycles per min. The digest was centrifuged at 500 *g* for 2 min, and resuspended in medium with 10% FCS. The incubation was repeated as described above. Cells were resuspended in 10 mL Waymouth's MB 752/1 medium and selectively sedimented by centrifugation at 100 *g* for 10 min rinsed twice with 20 mL of medium with 10 % serum. The pancreatic acinar cells obtained were resuspended in Waymouth's MB 752/1 medium buffered at pH 7.4 with bicarbonate and supplemented with 10% fetal calf serum (Life Technologies, New York, NY, USA) penicillin 100 U/mL and streptomycin 100 µg/mL at 37 °C in an atmosphere of 5% $CO_2$ and 95% air.

## Acinar Cell Cultivation

Cells were seeded at a density of $5x10^5$ cells /mL in a 35 mm plastic culture well in 2 mL of varied culture media and different supplements such as STI, BSA, dexamethasone and EGF (Sigma Chemicals, St. Louis, MO, USA) (Table 1). The viability of the acinar cells was estimated at specific time intervals using a 0.1% trypan blue (Sigma Chemical Co., St. Louis, MO, USA) dye exclusion test. The cultures showing 95% viability were used for further studies.

## Assessment of Amylase Activity

The determination of amylase activity was done using the method described by Bernfeld [19]. In brief, to determine the amylase activity of the supernatant (S) or of the homogenate (H), 0.25 mL of supernatant or homogenate were taken. To the tube containing supernatant and homogenate, 0.25

**Table 1.** Pancreatic acinar culture from BALB/c mice. Data represent 26 different cultures.

| Serial No. | Medium | Supplements | Viability and morphology on day 6 | Confluency on day 4 |
|---|---|---|---|---|
| 1. [1*, 24] | Waymouth's MB 752 with 10% FCS and antibiotics | HEPES 25mM | 55%, ++ | Same as seeded |
| 2. [1*, 24] | Waymouth's MB 752 with 10% FCS and antibiotics | STI 0.1 mg/mL<br>BSA 5 mg/mL<br>Dexamethasone $10^{-8}$M<br>EGF 4.2 nM | 85%, ++++ | Cells start attaching, spread after day 10. Passaged for 2 times. |
| 3. | DMEM (E) with 10% FCS and antibiotics | BSA 5 mg/mL<br>Insulin 5µg/mL<br>Transferrin 5µg/mL | 50%, + | Same as seeded |
| 4. [24*] | Ham's F-12 K with 10% FCS | BSA 5 mg/mL<br>Insulin 5µg/mL<br>Dexamethasone $10^{-8}$ mM<br>Transferrin 5 µg/mL | 70%, ++ | Same as seeded |
| 5. [24*] | Ham's F12 K with 10% FCS | STI 0.1 mg/mL<br>BSA 5 mg/mL | 83%, +++ | Cells start attaching and spreading. Passaged for 3 times. |

* The landmark attempts made in the maintenance of acinar cell cultures.

mL of distilled water and 0.25 mL of triton X-100 (0.0625%) were added, respectively. To all tubes, 0.5 mL of starch was added, incubated for 3 minutes at 20 °C following the addition of starch, 1 mL of 5-dimethylaminonaphthalene-1-sulfonamide (DNSA) was added to each tube. To terminate the enzyme reaction, the tubes were kept in a boiling water bath for 5 min. The tubes were then cooled to room temperature. Twenty mL of distilled water were added to each tube. The absorbance was read at 550 nm spectrophotometrically. The amylase activity was then expressed in terms of mg of maltose from a standard graph of optical density *versus* concentration of maltose. This standard graph was plotted by adding 1 mL of DNSA to different dilutions of maltose ranging from 0.2 to 2 mg/mL. From the third portion of the homogenate, the protein content of the tissue was determined using Bradford's method [20].

**Response to Secretagogue**

As a measure of acinar function, amylase production was measured on days 1 and 7 in unstimulated and carbamylcholine-stimulated cells. For studies of acinar cell response to secretagogue, the medium was aspirated and discarded and the remaining tissue was transferred to a conical centrifuge tube. Ten mL were pipetted (large bore to avoid mechanical shearing) and centrifuged at 1000 rpm for 10 min. The pellet was resuspended in fresh media with $10^{-5}$ M carbamylcholine (without serum). This was incubated for 30 min at 37 °C. The media and cells were transferred to fresh tubes and centrifuged; the supernatant was collected and amylase activity was measured in terms of μM/min/mg protein. All samples were processed in triplicate and an average was taken.

**Amylase Estimation**

The amylase activity was determined using the method of Bernfeld [19]. The absorbance was read at 550 nm by a spectrophotometer (U-3210, Hitachi, Tokyo, Japan). The amylase activity was expressed in terms of mg of maltose from a standard graph of optical density *versus* concentration of maltose.

**Protein Estimation**

The protein content of the tissue was determined using Bradford's method [20].

**Microscopy**

Acinar cell cultures were observed using a phase contrast inverted microscope (Olympus Tokyo, Japan) and were then photographed.

## ETHICS

The local animal research committee approved the experimental protocol. The animals used were cared for in accordance with the principles of the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences (NIH publication 86-23 revised 1985).

## STATISTICS

Amylase activity is reported as mean±SD. Data were analyzed by means of the unpaired t-test. Two-tailed P values less than 0.05 were considered statistically significant.

## RESULTS

The acinar cells are highly differentiated cells with very little capacity of proliferation. The literature on maintenance of acinar cells *in vitro* indicates that these cells remain viable and functional for only 4 days. It is essential to maintain acinar cells for long term and hence this study was undertaken to define the conditions necessary for long-term maintenance of differentiated exocrine acinar cells *in vitro*. One million cells were routinely plated in a 16 mm plastic culture well in 2 mL of various media (Table 1). The investigation was divided into two parts. First, dissociation conditions were defined in such a way as to yield the highest number of differentiated

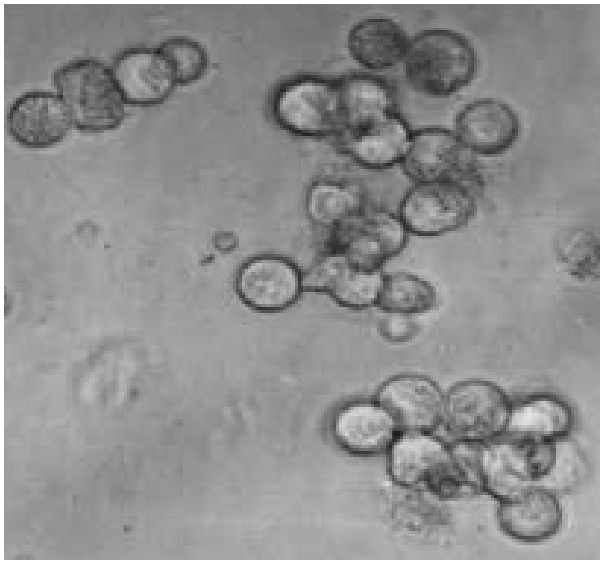

**Figure 1.** Freshly isolated pancreatic acinar cells. Phase contrast light microscopy. Magnification x 20.

viable acinar cells. The 0.2 mg/mL BSA which was used in the digestion medium helped acini to remain viable and differentiated for a longer period. Second, culture conditions were determined which would extend the viability of these cells far beyond certain period of time, retaining their secretory responsiveness in terms of amylase activity.

## Effects of Media

Pancreatic acinar cells were cultured in different culture media as described in Table 1. The tissue preparation consisted of one to several acinar units (Figure 1). Ductal fragments and islets of Langerhans were rarely seen. Cellular viability of freshly isolated acini was around 90%. The viability decreased to 55%, 50% and 70% for culture media 1, 3 and 4, respectively. On day 6, the percent viability of acini was 85% and 83% in media 2 and 5, respectively. In culture media 1, 3 and 4, the larger acinar fragments, underwent necrotic changes during the first 6 days in culture (Table 1). In media 1 and 4, the cells were less degenerated than in medium 3. There was no attachment seen in any of these three media. The remaining cells were relatively normal in appearance. In media 2 and 5, more than 80% of the acinar cells maintained a normal appearance at 6 days in culture and also started attaching and spreading as a monolayer (Figures 2a, 2b, and 3). The normal-appearing acinar cells varied in size and shape.

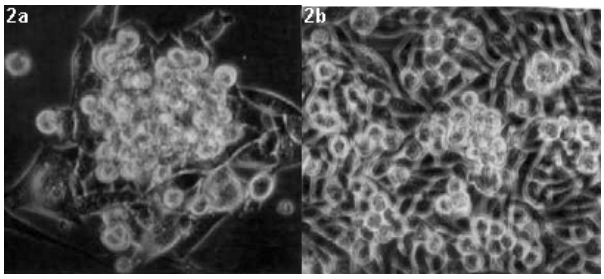


**Figure 2.** Cultured pancreatic acinar cells, 48 hrs cultures. Phase contrast light microscopy of pancreatic acinar cells which have reaggregated into multicellular clusters. Magnification x 40.

## Effects of Secretagogue

Of the various media used, culture media 2 and 5 showed the maximum amylase activity and viability after 7 days; therefore they were chosen for further study. Biochemical analysis comparing amylase contents in the cells cultured with or without secretagogue confirmed the morphological observations. Carbamylcholine induced an increase in intracellular amounts of amylase as well as in its release when compared with unconditioned cells. At the time of isolation, dispersed acini in control or grown in medium 5 responded to

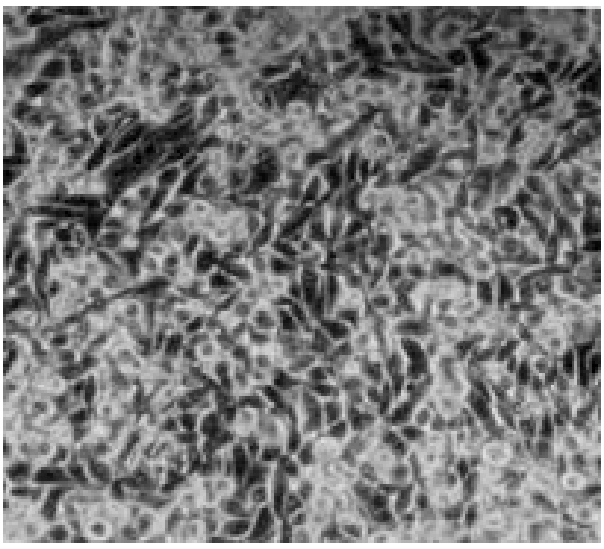

**Figure 3.** Phase contrast microscope image of a spreading colony of a primary culture of mouse pancreatic acinar cells as a monolayer on a plastic dish. Magnification x 20.

**Table 2.** Secretory responsiveness of freshly isolated and cultured acinar cells in media 2 and 5 (Mean values±SD).

| Sample | Amylase activity (μM/min/mg protein) | Viability |
|---|---|---|
| **Medium 2** | | |
| **A. Freshly isolated** | | |
| Basal (No. 8) | 0.257±0.200 | 98.1% |
| $10^{-5}$ M carbamylcholine (No. 9) | 0.831±0.220 | |
| | t=5.63; P<0.001 | |
| **B. 7 day old culture** | | |
| Basal (No. 10) | 0.036±0.021 | 83.0% |
| $10^{-5}$ M carbamylcholine (No. 9) | 0.061±0.032 | |
| | t=1.99; P=0.063 | |
| **Medium 5** | | |
| **A. Freshly isolated** | | |
| Basal (No. 8) | 0.319±0.230 | 98.4% |
| $10^{-5}$ M carbamylcholine (No. 10) | 0.839±0.110 | |
| | t=5.88; P<0.001 | |
| **B. 7 day old culture** | | |
| Basal (No. 9) | 0.042±0.014 | 80.4% |
| $10^{-5}$ M carbamylcholine (No. 9) | 0.077±0.025 | |
| | t=3.66; P=0.002 | |

carbamylcholine with a 3.23 to 4.15 fold increase in amylase secretion in 60-min period as compared to non-stimulated secretion in the same period (Table 2). After 7 days in culture, acinar cells still responded to carbamylcholine with a 2.60 fold increase in amylase secretion as compared to non-stimulated secretion in culture medium 5; a 1.69 fold increase was seen in medium 2. The percent viability on the 7th day in the control was 80.1% as compared to 80.4% in medium 5 (Table 2). This indicated that medium 5 permits maintaining acinar cells for more than 4 days, in terms of viability and functionality.

## DISCUSSION

In this investigation, we have described a simple method of establishing primary cultures of murine acinar cells with maximum viability and retention of amylase secretory activity for a period of 7 days. We have modified and improved the dissociation procedures. Successful cultivation of acinar cells depends upon the consistent and uniform isolation of pancreatic acinar cells by collagenase digestion. Numerous factors contribute to the poor and inconsistent yield of acinar cells. The extreme variability in activity of collagenase preparations represents a major obstacle to the successful isolation of these cells. Moreover, the variable degree of activation of native proteolytic enzymes within the acinar cells which occurs during digestion contributes to the variability of the results reported. The factors which allow acinar cell adaptation and death may be due to differences in the nutritional constituents such as medium content of amino acids, glucose, pH and salts in buffers, lipids present in the BSA preparation or oxygenation condition [21]. Based on previous studies which ascribe an important role to secretagogues *in vitro* [5, 6], experiments were performed to investigate their mechanism of action. The present study demonstrates that acini may be protected by soybean trypsin inhibitor (Table 1). The premature activation of zymogens within the acinar cell may be an early event in digestion of the cells. Thus the use of complete medium supplemented with soybean trypsin inhibitor, BSA, dexamethasone and EGF possibly helps in preserving cell integrity and viability, as is demonstrated by the present study.

The secretory responsiveness to carbamylcholine is maintained in cultured cells and exhibits a similar magnitude of stimulation of secretion. It is well-known that dissociated acinar cells are not as responsive as intact acini to secretagogue [1]. In spite of

the magnitude of the secretagogue-response sustained in cultured cells, the absolute secretion of amylase by cultured cells decreases approximately 7.5-8% of the absolute secretion as compared to freshly isolated cells. Thus, both basal and stimulated amylase secretion by freshly isolated and cultured acinar cells represent a similar percentage of the total amylase content released by these respective cells. The amylase secretion pattern obtained in our study resembles that reported by Zhao *et al.* exhibiting high levels of amylase from freshly seeded acinar cells with a gradual decrease in enzyme activity with time [22]. Protease inhibitors have been used to reduce the severity of acute pancreatitis by blocking the activity of active enzymes that are generated within the pancreas or released systemically. Although benefits from protease inhibitors have been reported in humans and experimental models, this therapy has not been consistently beneficial. One of the features of cerulein induced acute pancreatitis in rats was the formation of large cytoplasmic vacuoles within the acinar cells which occasionally contained small aggregates of electron-dense material. Exogenous protease inhibitors have been reported to prevent cerulein-induced pancreatitis and reduce vacuole formation in rats [23].

In summary, our study demonstrates that acinar cells cultured in media containing STI increase their viability in culture medium and consequently reduce the rate of death. This may be due to both the inhibition of protease within the secretory pathway and the inhibition of the extracellular trypsin released during the death of acinar cells, thus indirectly protecting the remainder of the cells. It also helps the acinar cells to form a monolayer with hormonal responsiveness. The present study indicates, moreover, that all the given parameters play an important role in preventing acinar cell injury which is probably the root cause for such the life of acinar cells studied *in vitro*. It can be inferred from the study that maintenance of the functional state of the acinar cells *in vitro* is controlled by the composition of the nutrient media which may differ from their *in vivo* counterparts.

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**Key words** Amylases; Cell Survival; Nutrition; Pancreas

**Abbreviations** BSA: bovine serum albumin fraction V; DNSA: 5-dimethylaminonaphthalene-1-sulfonamide; FCS: fetal calf serum; STI: soybean trypsin inhibitor

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