

Evaluating prostate cancer cell culturing methods: A comparison of cell morphologies and metabolic activity

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Abstract. LNCaP prostate cancer cells were grown under four unique cultivation conditions. Two types of bioreactor systems were used to observe the influence of low-shear culture conditions allowing for three-dimensional growth: a) a perfusion rotating wall vessel (RWV) bioreactor; and b) a high aspect ratio vessel (HARV) RWV bioreactor, with periodic medium exchanges (fed-batch). In addition, two growth methods utilized tissue culture flasks (TCFs): a) unaltered or conventional TCFs; and b) poly(2-hydroxyethyl methacrylate) [poly(HEMA)] coated TCFs, to inhibit cell attachment. Comparisons were drawn based on qualitative observation of cell morphology and quantitative metabolic data. Similar cellular metabolism was demonstrated for cells grown under each condition. The degree of aggregation, however, varies considerably. Spherical shaped aggregates with diameters of 1 to 3 mm were produced when cells were grown within the perfusion-RWV bioreactor. All other growth conditions produced irregular shaped aggregates of various sizes. Quantitative results demonstrated the expected glucose utilization concomitant with lactate accumulation. Immunohistochemical evaluations were unremarkable for all four cultivation conditions. Results demonstrate that use of the perfusion-RWV bioreactor is advantageous in obtaining spherical aggregates, while grown in a controlled environment.

Introduction

The rotating wall vessel (RWV) bioreactor system developed by NASA has been reported to simulate microgravity

conditions for cell growth purposes. RWVs maintain cells in a suspended state with minimal shear forces, thereby allowing cells to form three-dimensional aggregates (1-12).

RWVs consist of a cylindrical chamber completely fluid-filled and bubble-free, that operate on the principle of solid body rotation about a horizontal axis. This configuration results in a 'no-slip' condition at the wall of the chamber, thereby allowing the chamber and culture fluid to rotate at essentially a constant angular velocity. Suspension of the cells is achieved by varying the angular velocity of the system to counteract the sedimentation velocity of the particles due to gravitational forces. This process results in a continuous state of free fall, which provides low-shear stress (1,2,4,7,13). The combination of suspended cells and bubble-free aeration leads to the environment of low normal and shear forces that foster three-dimensional-spheroid growth and cell differentiation. A well-controlled growth environment, e.g., maintaining relatively constant nutrient concentrations and minimizing byproduct accumulation, can be obtained either by periodic medium exchange or by perfusing the system with fresh medium.

As cellular structures and cell-cell interactions have been shown to play a role in cell growth and differentiation, the bioreactor culture system more closely mimics the *in vivo* environment. The growth and organization of several human carcinoma cell lines on microcarriers have been studied using bioreactors (8-10,14-19). Additionally, some co-cultures with these cell lines have been studied (10,15,19). Cells were found to organize into aggregates, displaying cell-line specific morphologies ranging from spheroids of varying compactness to structures resembling folded sheets of tissue. Overall, the published literature suggests that cell culture in the RWVs presents a useful method for the modeling of some *in vivo* characteristics of human carcinoma.

The purpose of the experiments described here was to examine differences in four distinct cell cultivation conditions for LNCaP prostate cancer cells. LNCaP cells were grown in conventional, untreated tissue culture flasks (TCFs), poly(2-hydroxyethylmethacrylate) [poly(HEMA)] coated TCFs, a fed-batch high aspect ratio vessel (HARV) RWV bioreactor, and a perfusion-RWV bioreactor. We evaluated cell aggregation; glucose consumption, lactate production, carbon dioxide,

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oxygen, and pH were monitored; and finally, hematoxylin-eosin staining, and CD44, E-cadherin, and prostate specific antigen (PSA) expression were determined.

Materials and methods

Cell culture. LNCaP.FGC (ATCC CRL 1740), a human prostatic carcinoma cell line originally derived from a lymph node metastasis (20), was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 growth medium (Gibco BRL, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 10 mM HEPES (Bio Whittaker, Walkersville, MD), 1 mM sodium pyruvate (Gibco BRL, Rockville, MD), 100 U/ml penicillin-streptomycin (Gibco BRL, Rockville, MD), 1.9 mM L-glutamine (Gibco BRL, Rockville, MD), 0.05 mg/ml gentamycin (Cellgro, Herndon, VA), and 0.1 mM 2-mercaptoethanol (Bio-Rad, Philadelphia, PA). In this study, cells for each culturing method were prepared from the same cell passage. All cultures were grown in humidified incubators (Fisher Scientific, Pittsburgh, PA) at 37°C and 5% CO₂.

Cultivation systems

Conventional tissue culture flasks. Fifteen untreated polystyrene 75 cm² TCFs were used and provided a surface for the cells to adhere to, thereby creating a system of two-dimensional growth under the influence of gravity.

Poly(HEMA) coated tissue culture flasks. Fifteen 75 cm² TCFs were coated with poly(HEMA). The coating procedure for the TCFs was adapted from previous work (21). Briefly, a stock solution of poly(HEMA) (Sigma Chemical, St. Louis, MO) was produced by dissolving poly(HEMA) in 95% ethanol at 120 mg/ml with vigorous shaking and incubation at 37°C overnight. Each flask was coated with a 1:10 dilution of this poly(HEMA) solution in 95% ethanol. Of the diluted solution, 7.5 ml was added to each 75 cm² TCFs and allowed to air dry in a laminar flow hood until the following day. The poly(HEMA) coating inhibited cell adherence, thereby promoting three-dimensional growth under the influence of gravity.

Fed-batch HARV RWV bioreactor. The HARV RWV bioreactor system was purchased from Synthecon, Inc. (Houston, TX). This unit consists of a 50 ml volume reactor vessel that rotates along a central axis. Oxygen is provided through silicone membrane on one face of the rotating reactor chamber. A detailed description is published elsewhere (5,9,22,23). A daily exchange of medium, consisting of an exchange of half of the total volume for fresh medium, was conducted every 24 h after initial inoculation.

Perfusion-RWV bioreactor. The perfusion-RWV bioreactor was also obtained from Synthecon, Inc. (Houston, TX). This unit consists of a 150 ml volume reservoir for cell cultivation. An in-line oxygenator coupled with an axial silicone membrane provides oxygen. Further details are published elsewhere (15,23,24). The perfusion rate was 0.35 ml/min, which replaced 1.5 times the total system volume every 24 h.

Inoculation procedures and maintenance. For all cultivation methods, a pH adjustment protocol was used to allow for equilibration between the medium and the growth vessels. Twelve hours prior to inoculation, all vessels were filled with growth medium and allowed to sit overnight in the incubators. Just prior to inoculation, this medium was replaced with fresh medium.

The TCFs were each inoculated with 2.78x10⁵ cells/ml. A new flask was sampled each day and all flasks were maintained for the duration of the experiment (96 h). The poly(HEMA) coated TCFs were also inoculated with 2.78x10⁵ cells/ml and maintained for the duration of the experiment. The fed-batch HARV RWV bioreactor was inoculated with 3.20x10⁵ cells/ml. Sampling from the HARV RWV bioreactor was performed both before and after the daily exchange of medium, since half of the total volume of medium was exchanged for fresh medium every 24 h. Each of these experiments was conducted in triplicate and lasted 96 h. The perfusion-RWV bioreactor inoculation density varied with experiment. Specifically, inoculation densities were 3.3x10⁵, 2.0x10⁵, and 1.8x10⁵ cells/ml for experiments 1, 2, and 3, respectively. When sampling from the perfusion-RWV, fresh medium was added to the growth chamber to compensate for the amount removed for each sample. Each cultivation in the perfusion-RWV was maintained for a period of 96 h. Both bioreactors required that the vessel rotation be stopped in order to sample from the growth chambers.

After inoculation, the rotation rate was empirically adjusted to compensate for the increased sedimentation rate as the cell aggregates grew. The rotation for the HARV RWV was started at 9.0 rpm and ended at 13.0 rpm. The rotation rate for the perfusion-RWV varied with aggregate size. Rotation was started at 8.5, 5.0, and 5.0 rpm and ended at 11.8, 6.2, and 6.2 rpm for experiments 1, 2, and 3, respectively.

Sampling procedures. An aliquot of growth medium from each chamber was collected at 24-h intervals after inoculation. All samples gathered for glucose, lactate, and PSA expression analysis were collected in sterile Luer lock syringes (Becton Dickinson, Franklin Lakes, NJ), passed through a 0.22 µm syringe filter (Millex®-GS, Millipore Corporation, Bedford, MA) into an Eppendorf tube and kept frozen at -15°C until analysis. Prior to analysis, samples were warmed to room temperature. All samples gathered for oxygen, carbon dioxide, and pH analysis were collected in untreated 3 ml Vacutainers® (Becton Dickinson, Franklin Lakes, NJ), placed on ice, and analyzed the same day as collection.

Analytical measurements. Glucose and lactate concentrations were determined with a YSI model 2300 STAT plus analyzer (YSI Inc., Yellow Springs, OH). The levels of oxygen, carbon dioxide, and pH were measured with a Radiometer Copenhagen Blood Gas Analyzer (West Lake, OH) in the Clinical Chemistry Laboratory at the University of Iowa Hospitals and Clinics. Prostate specific antigen (PSA) was measured by a micro-particle enzyme immunoassay (MEIA), performed on an IMx system (Abbott Diagnostics, Abbott Park, IL).

Immunohistochemical procedures. Hematoxylin-eosin (H&E), E-cadherin, and CD44 were determined by immunohisto-

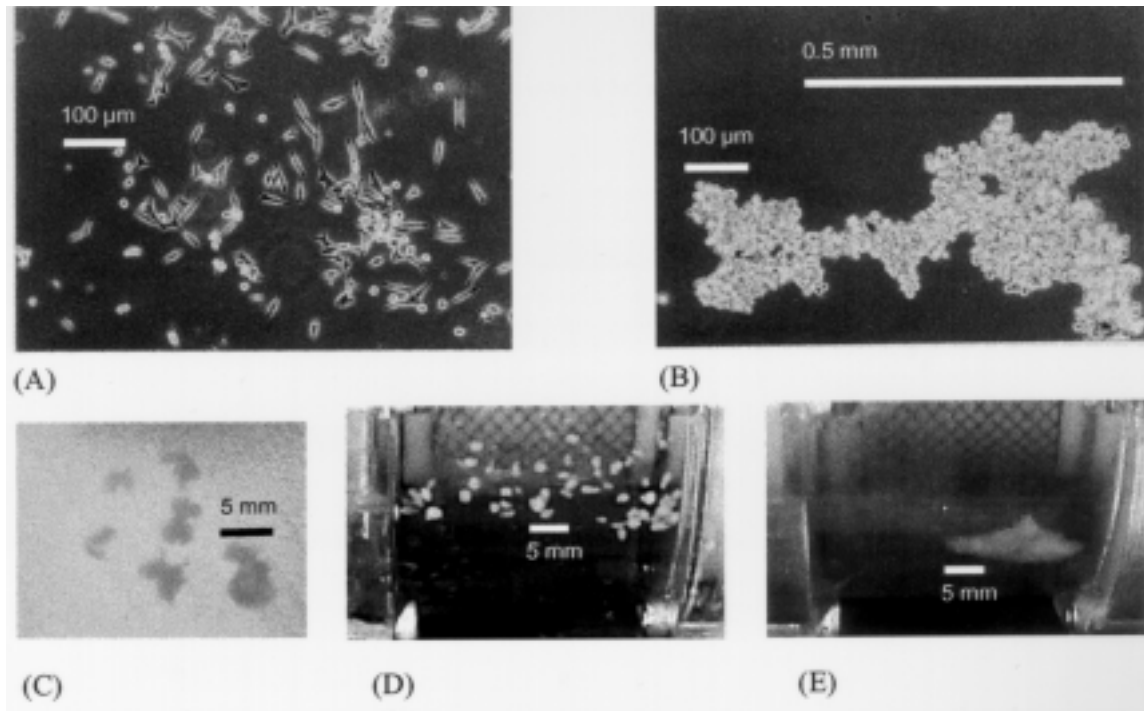


Figure 1. Photographs of typical LNCaP cells after 48 h of cultivation in: (A) conventional TCFs; (B) poly(HEMA) coated TCFs; (C) fed-batch HARV RWV bioreactor; (D) perfusion-RWV bioreactor; and (E) perfusion-RWV bioreactor. Rotation of the vessel was stopped for the fed-batch HARV RWV bioreactor and cell aggregates were permitted to settle before the photograph was taken. Photographs taken from the perfusion-RWV were taken as the reactor rotated. Aggregation patterns demonstrated in (D) and (E) correspond to patterns observed when the rotation started immediately following inoculation and after 5 to 30-min post inoculation, respectively.

chemical methods. Cell aggregates from each culture method were fixed in formaldehyde (Sigma Chemical, St. Louis, MO). The paraffin-fixed cell blocks were then cut into 3 µm slices with a rotary Leitz 1512 microtome (Henry Louis, Iowa City, IA). For all samples, sections were deparaffinized in xylene (Fisher Scientific, Pittsburgh, PA), rehydrated in graded ethanols, and rinsed, using standard protocol. Proteolytic digestion was accomplished using trypsin (Gibco, Grand Island, NY) for 15 min at 37°C. Antigen unmasking was accomplished by incubating slides in citric acid buffer solution (Biogenex, San Ramon, CA) in a microwave for 2-5 min cycles on high power. Slides were allowed to cool to room temperature.

H&E staining was accomplished using standard protocol. A 10% harris hematoxylin (Surgipath Co., Richmond, IL) solution was used. Hematoxylin was added to each section for 2 min, rinsed with deionized water, rinsed with tap water (to allow the stain to develop), rinsed with acid ethanol (to destain), rinsed with tap water, and then rinsed with deionized water. The excess water was removed and then the eosin staining was accomplished. Eosin was added to each section for 30 sec, then rinsed with graded ethanols, and finally rinsed with xylene. The slides were left overnight in xylene to ensure good clearing of the ethanol.

Specifically, an immunoperoxidase reaction for E-cadherin (Sigma Chemical, St. Louis, MO) and CD44 (Becton Dickinson, San Diego, CA), was performed by horseradish peroxidase labeling method (25) (Dako, Carpinteria, CA). Endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide in distilled water. Non-specific back-

ground staining was prevented by application of non-immune horse serum (Vector, Burlingame, CA). Sections were covered with anti-E-cadherin and CD44 antibodies (Dako). Both primary antisera were used at a dilution of 1:50, incubated overnight at 4°C, and rinsed. Sections were then covered with labeled streptavidin biotin (LSAB) biotinylated antibody (Dako) for 20 min at room temperature, rinsed, and then covered with LSAB streptavidin (Dako) for 20 min at room temperature. The sections were then rinsed and incubated with diaminobenzidine (Aldrich, Milwaukee, WI) to demonstrate the signal of the primary antibody. Negative control slides were prepared by substituting PBS.

Results

Morphological observations. Cell morphology was highly dependent on the culturing method (Fig. 1). Cells grown in conventional TCFs grew two-dimensional, adhering to and covering the TCF surface (Fig. 1A). The poly(HEMA) coated TCFs resulted in the formation of three-dimensional rod-like aggregates (1-3 mm) in suspension (Fig. 1B). In the fed-batch HARV RWV, irregularly shaped three-dimensional aggregates (2-7 mm) were formed in suspension (Fig. 1C). In the perfusion-RWV the number and size of the aggregates were dependent on how soon the rotation was started after inoculation. When rotation was not started immediately after inoculation, the cells aggregated together within minutes to form a single three-dimensional aggregate (2x1 cm) (experiment 1) (Fig. 1E). The adhesion forces between cells were strong enough that increasing the rotation speed did not

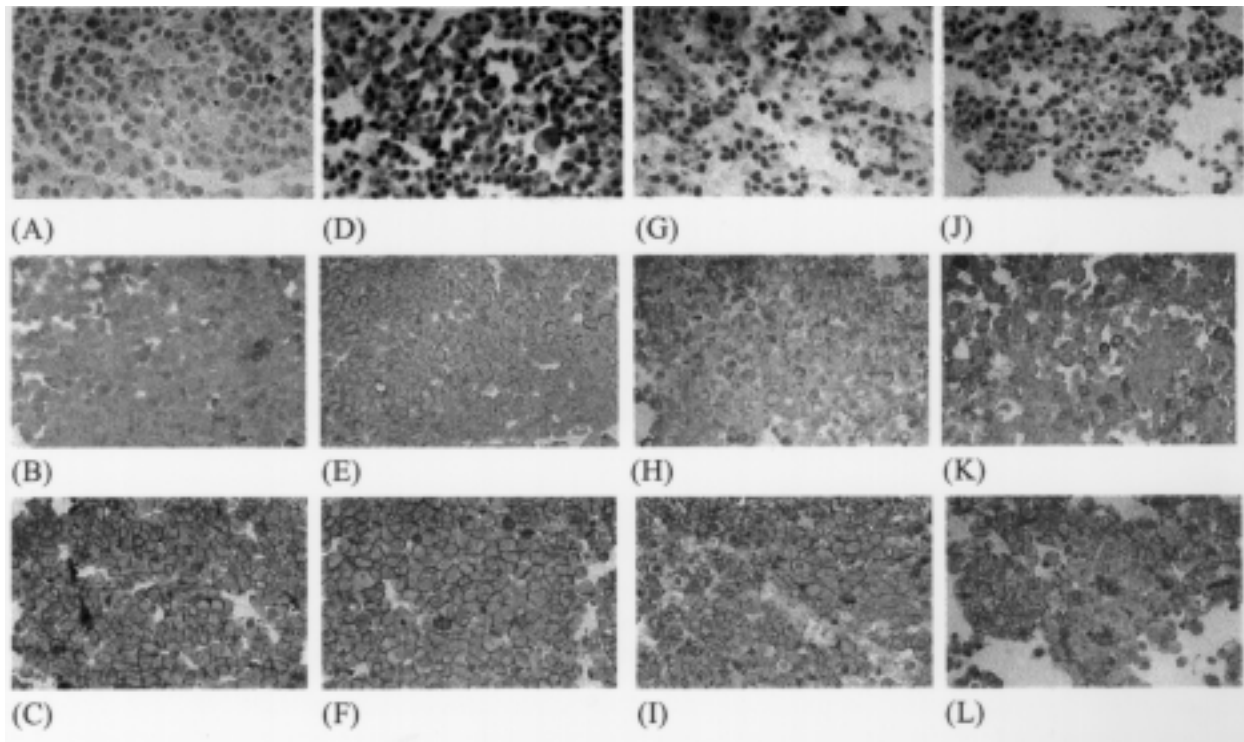


Figure 2. Immunohistochemical photomicroscope images from four different cultivations. Specifically, H&E (A), CD44 (B) and E-cadherin (C) for the conventional TCFs; H&E (D), CD44 (E) and E-cadherin (F) for the poly(HEMA) coated TCFs; H&E (G), CD44 (H) and E-cadherin (I) for the fed-batch HARV RWV; H&E (J), CD44 (K) and E-cadherin (L) for the perfusion-RWV. Magnification for all images, $\times 40$.

disrupt the aggregate. In contrast to these results, 35 to 55 spherical aggregates (1-3 mm) were formed when rotation was initiated immediately following inoculation of culture (experiments 2 and 3) (Fig. 1D). It is also important to note that necrotic cell death was observed within the single large aggregate, but not in the smaller aggregates produced in the perfusion-RWV.

H&E, CD44, E-cadherin, and PSA. No differences were noted in cell morphology when evaluated by H&E staining (Fig. 2). Additionally, no significant differences were observed in the pattern of expressed proteins in cells grown under these four conditions. No CD44 was evident in any of the cultivation cell groups (Fig. 2). This is consistent with previous results obtained in TCFs (26,27). E-cadherin was expressed in all groups of cells with no statistically significant differences in the amount of E-cadherin expressed (3-, 3-4+, 3+, and 3-4+, for the conventional TCFs, poly(HEMA) coated TCFs, fed-batch HARV RWV, and perfusion-RWV, respectively) (Fig. 2). Similarly for PSA levels, no significant differences in expression were discernable in the various cell growth conditions (data not shown).

Glucose, lactate, and pH. Glucose and lactate concentrations and pH were determined every 24 h for a period of 96 h for all growth methods (Fig. 3). Glucose consumption, lactate accumulation, and pH followed similar trends in the conventional and poly(HEMA) coated TCFs (Fig. 3A and B). For the conventional and poly(HEMA) coated TCFs, glucose concentrations decreased from 10.39 ± 0.41 to 8.40 ± 0.29 mM

and 10.21 ± 1.0 to 7.57 ± 0.82 mM, respectively (95% confidence levels, $n=11$), lactate concentrations increased from 1.47 ± 0.07 to 4.12 ± 0.49 mM and 1.44 ± 0.15 to 5.66 ± 1.23 mM, respectively (95% confidence levels, $n=11$), and the pH remained relatively stable at 7.17 ± 0.04 and 7.16 ± 0.04 , respectively (95% confidence levels, $n=5$). Glucose and lactate concentrations and pH are shown for the fed-batch HARV RWV in Fig. 3C. The glucose concentrations decreased over each 24-h period and decreased from 11.02 ± 0.55 to 9.47 ± 0.31 mM (95% confidence levels, $n=7$) over the length of the experiment. Lactate concentrations increased over each 24-h period and increased from 1.54 ± 0.09 to 4.91 ± 0.92 mM (95% confidence levels, $n=7$) over the length of the experiment. The pH was relatively constant over the course of the experiment (7.25 ± 0.04 , 95% confidence levels, $n=3$). The results for the perfusion-RWV (Fig. 3D) can be broken into two sections, i.e., the first 48-h and the last 48-h. During the first 48-h time period, medium glucose levels were depleted (10.78 ± 0.41 to 9.26 ± 0.74 mM), lactate levels were elevated (1.64 ± 0.10 to 4.02 ± 0.73 mM), and the pH decreased (7.2 ± 0.1 to 6.5 ± 0.1) (95% confidence levels, $n=7$, $n=7$, $n=3$, respectively). During the last 48-h time period, glucose, lactate, and pH levels began to stabilize (9.70 ± 0.36 mM, 3.28 ± 0.17 mM, and 7.02 ± 0.03 , respectively, (95% confidence levels, $n=7$, $n=7$, $n=3$, respectively).

Yield coefficients (28) were calculated for each of the cultivation conditions. These coefficients correspond to the ratio of the moles of lactate produced over the moles of glucose consumed for the 4-day growth period. Yield coefficients were 1.00 ± 0.85 , 1.25 ± 0.25 , 1.47 ± 0.25 , and 1.78 ± 0.26 for cells

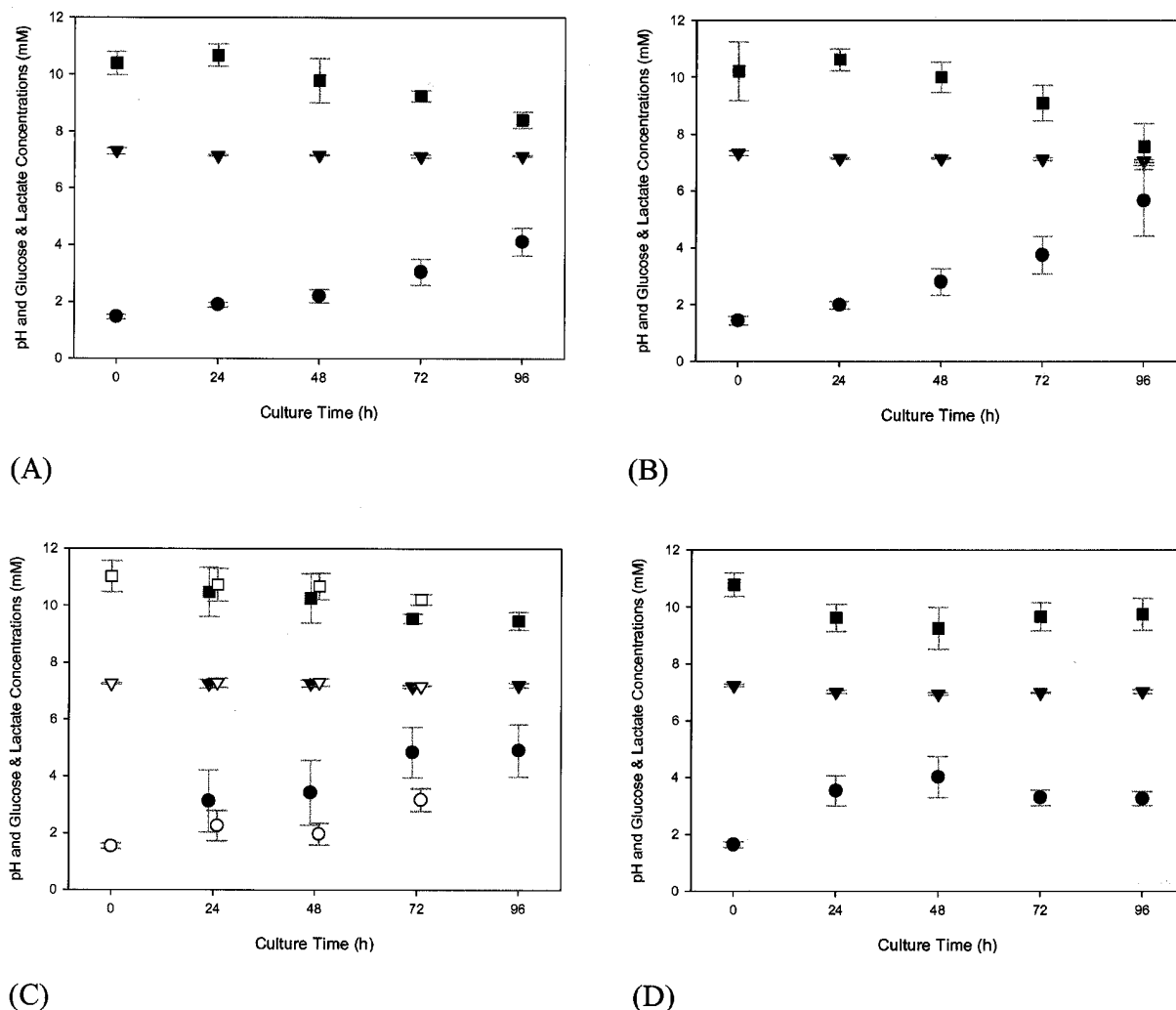


Figure 3. Average pH (\blacktriangledown), glucose (\blacksquare) and lactate (\bullet) concentrations of LNCaP cancer cells over a period of 96 h in four different growth conditions are shown above: conventional TCFs (A); poly(HEMA) coated TCFs (B); fed-batch HARV RWV bioreactor (C); and perfusion-RWV bioreactor (D). Half of the medium was exchanged in the fed-batch HARV every 24 h. Samples that were collected just prior to the medium exchange are shown with closed symbols (\bullet) and samples collected just after the medium exchange are shown with open symbols (\circ). The error bars represent the 95% confidence limits based on the given total number of individual observations (n): (A) and (B) n=5 for pH, n=11 for glucose and lactate; (C) and (D) n=3 for pH, n=7 for glucose and lactate.

grown in conventional TCFs, poly(HEMA) coated TCFs, the fed-batch HARV RWV bioreactor, and the perfusion-RWV bioreactor, respectively. These values correspond to the mean (\pm standard deviation) for three independent cultivation runs. In addition, carbon dioxide and oxygen levels were unremarkable for all four cultivation methods.

Discussion

The purpose of this investigation was to study the effects of culturing conditions on various characteristics of LNCaP cells. Specifically, the metabolism and morphology of cells grown in conventional TCFs, poly(HEMA) coated TCFs, a fed-batch HARV RWV, and a perfusion-RWV were compared. From the results discussed above, it is clear that the perfusion-RWV is well suited for satisfying the two key criteria for simulating *in vivo* conditions, i.e. a well-controlled environment (when used with a monitoring/control system) and three-dimensional cell growth. It is worth noting, however, that

it is questionable whether most (if not all) existing cancer cell lines mimic actual conditions in the human body due to the methodologies used in isolating cell lines from tissue and the culturing thereafter (29). It is likely that the same is true for cell lines that purportedly represent other *in vivo* conditions.

Major differences were observed in the morphology and aggregation of the cells. Conventional TCFs resulted in expected monolayer of cells strongly adhering to the flask surface and appear two-dimensional. The poly(HEMA) coated TCFs, resulted in the formation of three-dimensional irregular-shaped rod-like aggregates in suspension, as expected. Aggregates of this type are well known (21,30,31). Both the fed-batch HARV RWV and perfusion-RWV bioreactors resulted in three-dimensional aggregate formation. Aggregates of cells in the fed-batch HARV RWV bioreactor were irregularly shaped and non-uniform in size. Aggregates varied in size between 2 and 7 mm at 96 h. Different size aggregates were obtained with each bioreactor run and

typically, the number of aggregates ranged between four and seven for each cultivation.

Aggregation in the perfusion-RWV bioreactor was highly dependent on the procedure used to initiate reactor rotation. Specifically, many small aggregates formed when vessel rotation was started immediately following inoculation of cells. Typically, 35-55 spherical aggregates formed with an average diameter of 1 to 3 mm at 96 h. On the other hand, one large aggregate formed when rotation was started 5-30 min after inoculation. In this case, the aggregate was irregularly shaped with approximate dimensions of 2x1 cm at 96 h. An attempt was made to disperse this single large aggregate by increasing the vessel rotation speed and, thereby, increasing shear stress forces on the cells. This attempt failed. The aggregates produced from the perfusion-RWV system varied in size depending on when rotation was initiated. Since it was desirable to create a system that produced similar aggregates for all systems evaluated, the rotation was initiated immediately after inoculation of cells. This proves to be an advantage for other investigations. Aggregate size and shape can be varied for LNCaP cells with the perfusion-RWV system. Though cell growth only seemed to occur within the first 48 h for the perfusion system and thereafter the cells are maintained for the next 48 h. Overall, the results for all systems indicated growth. We are continuing to evaluate the perfusion-RWV growth method. Initially, we attempted to quantify the yield of cells from the bioreactors, but this proved to be unsuccessful. Cells growing in spheroids were damaged or destroyed during the removal process. This process is still under investigation.

There is a small and directly relevant literature in prostate cancer research (5,32-40). A variety of different cell lines have been studied and using a variety of methodologies to attain spheroid growth. In several of these studies, the end point has been an assessment of the response to various therapeutic agents, particularly chemotherapeutic agents. At least two groups have looked at PSA production (39,40). In addition, there is at least one report regarding CD44 expression (39). Given the different methodologies used, it is difficult to identify biologically meaningful results that have been described when compared to our work. For example, Lang *et al.* (39) reported differences in CD44 expression for PC3 cells grown in Madrigal®. In contrast, we did not identify any difference in expression in CD44 in LNCaP amongst any of the four growth conditions.

The degree to which the conventional TCF, poly(HEMA) treated TCF, fed-batch HARV RWV, and the perfusion-RWV cultures altered the medium were similar. It is important to mention here that each vessel should be filled with medium at least 12 h prior to inoculation with cells and fresh medium. This allowed for more control of initial glucose, lactate, and pH value. As expected, the greatest changes in glucose and lactate concentrations were seen in the two TCF systems. Since half of the medium was changed daily for the fed-batch system, it was expected that the changes would be less significant. Instead the fed-batch HARV RWV system was less predictable for these values than the perfusion-RWV system. This may be due to the modified medium exchange for the fed-batch HARV RWV. In addition, pH, carbon dioxide, and oxygen levels were unremarkable for all four

cultivation methods. Furthermore, the yield coefficients for the conventional TCFs and the poly(HEMA) treated TCFs were as expected. With the yield coefficients ideally being 2 for the conversion of glucose to lactate, the yield coefficients for these two schemes show an inefficient use of glucose from the medium. Since the medium was exchanged for both the fed-batch HARV RWV and perfusion-RWV bioreactors, the yield coefficients were expected to be higher. The yield coefficient was lower than expected and less predictable for the fed-batch HARV RWV than for the perfusion-RWV. Implementing a control scheme could significantly reduce the variations observed in the perfusion-RWV. For example, control of glucose and minimization of lactate accumulation could be based on a near-infrared spectroscopic sensor (41). Our laboratories are currently working on developing this technology for the real-time, on-line sampling, monitoring, and control of the perfusion-RWV bioreactor.

In summary, the results indicate that little difference in the expression of cellular adhesion proteins is observed when growing only LNCaP cells in any of these examined growth chambers. The highest growth yield coefficients were observed with the perfusion-RWV bioreactor. This finding is consistent with greater control of the chemical environment during cultivation. In terms of aggregation, the perfusion-RWV bioreactor provided many large spherical aggregates (1-3 mm), as opposed to the irregular shaped three-dimensional structures produced in the HARV RWV bioreactor and poly(HEMA) coated TCFs.

The unique aspect of our findings focus on the fed-batch HARV RWV and perfusion-RWV systems that provide the advantage of low-shear forces (versus high shear in other systems), as well as a system that allows for more regular exchanges of medium. Both of these would appear to have relevance to studying human prostate cancer. In both RWV bioreactors there is space for growth, promotion of three-dimensional formation, and multiple aggregation. We believe that continuing studies will establish more reliable methods for the formation of three-dimensional aggregation, including co-culturing with mesenchymal cells.

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