Bioreactor Based Production of Functional Conditioned Medium for the Propagation of Undifferentiated Human Embryonic Stem Cells

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Abstract: Embryonic stem (ES) cells are characterized by their capacity for self-renewal and their pluripotency. A crucial prerequisite for a therapeutic use of human ES cells is the ability to expand them to large numbers without affecting their pluripotency. For many murine ES cell lines, leukaemia inhibitory factor suffices to prevent spontaneous differentiation. In contrast, the human ES cell lines available depend on feeder cells, i.e., coculture with mitotically inactivated mouse embryonic fibroblasts (MEFs) or treatment with conditioned medium (CM) derived from such cultures. This dependency on feeder cells is associated with batch-to-batch variations, difficulties in implementing large scale suspension culture of ES cells and potential carry-over of pathogens from the feeder to the ES cell population.

Key words: human embryonic stem cells, conditioned medium, feeder cells, bioreactor

1. INTRODUCTION

The bioreactor based production of conditioned medium (CM) has shown advantages: Completely controlled conditions minimize the batch-to-batch variations while the amount of generated CM is high enough to enable large scale ES cultivation processes with pathogen screened CM. Different cell types, as STO (ATCC CRL-1503) and primary murine fibroblasts, were cultivated on porous microcarriers in a 2 litre stirred and bubble-free aerated perfusion process. The produced CM was tested on human ES cells (H 9.2)

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over multiple passages while the differentiation and proliferation of these cells were used as a marker for the evaluation of the CM´s functionality.

2. MATERIAL AND METHODS

Feeder cells (primary mouse fibroblasts, CD1) were isolated from 13 days old mouse embryos. In order to cultivate these adherent cells in a continuously stirred bioreactor, micro- and macroporous CultiSpher-G microcarriers were used. Due to a lack of attachment and growth factors in the ES cell medium (Table 1) the carriers were colonized under serum containing conditions (10% FCS). Therefore the microcarriers (MCs) have to be washed several times before inoculation. The growth of the MEFs on the carriers was inspected with MTT & DAPI staining periodically.

Table 1. Composition of 2,5 litre ES cell medium.

Knockout serum replacement	500 mL
Knockout DMEM with pyruvate	2000 mL
Non essential aminoacids (1:100)	25 mL
L – glutamin	1 mM
2 – Mercaptoethanol	0,1 mM

During the cultivation in the bioreactor (Fig. 1) several parameters, e.g. concentration of glucose, lactate, ammonium and lactate-dehydrogenase were examined at regular intervals. The cell density was determined with trypan blue staining after enzymatic degradation of the carriers and with a crystal violet staining. After a short period of batch cultivation, the perfusion was started. The harvest containing dead cells and cell debris was sterile-filtered and screened for mycoplasma contamination using PCR. Afterwards the CM was stored frozen. Before the CM was used as full-media for ES cells growing on Matrigel™ coated petri dishes it was supplemented with basic fibroblast growth factor (4ng/mL). The differentiation level of the ES cell culture was proven over at least three passages. Functionality of the CM was determined by staining for alkaline phosphatase (AP), a cellular marker for differentiation.

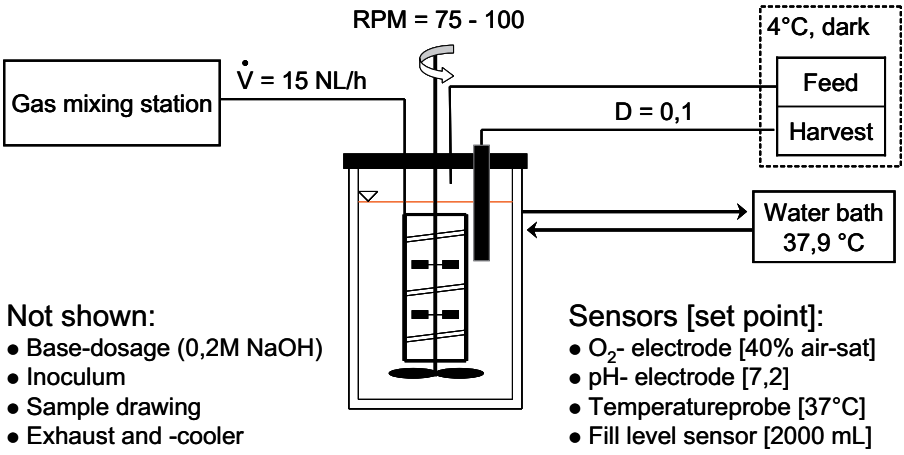

Figure 1. Schematic diagram of the 2 liter bioreactor used for the production of CM.

2.1 Inoculum

MEFs proliferate in the serum containing preculture-medium only. Because the CM should not contain complex animal components, the colonization of the MCs had to take place outside the bioreactor. Another consequence is, that no bead-to-bead transfer will occur in the bioreactor. To archive a cell density of at least 2 * 10^5 cells/mL, a preparation protocol for the preculture (Table 2) was developed. The colonization of the carriers was validated with MTT & DAPI staining.

Table 2. Protocol for the generation of the inoculum.

Day	Action
1	Thaw cells for 4 confluent T-175 flasks
2	Split cells to 12 T-175 flasks
4	Detach cells, add 5g (dry mass) prepared carrier
7-8	Detach carrier mechanically Wash carrier until serum concentration is less than 0,01% Inoculate the bioreactor, which should be in steady state

3. ARCHIEVED PROCESS DATA

Although there is no cell growth in the bioreactor the perfusion was started at day 4 in order to satisfy the maintenance demand of the MEFs. During the cultivation the concentrations of glucose and amino acids remained at a constant level. Because the pyruvate concentration decreased during the process we can assume that murine embryonic fibroblasts utilize pyruvate and glucose as carbon source. However, the substrate

concentrations were high enough to cover the specific uptake rates of ES cells. The concentrations of the measured metabolites lactate and ammonia were below 2 mM. The lactate-dehydrogenase activity was measured as a marker of the culture viability, it never exceeded 60 Units/L.

Another indication for the stationary phase of the cells is the pO_2 control point, which increased neither exponential nor linear but stayed constant over the whole cultivation time. Only during the first two days of cultivation a poor growth was observed, which can be explained with the memory effect of the cells.

4. RESULTS AND CONCLUSION

The goal to establish an easy to use, reproducible bioreactor process for generating great amounts of functional conditioned medium is nearly reached. The tests of the produced CM showed that the pluripotency of the ES cells can be maintained. In comparison, the functionality of the CM produced during the bioreactor process is at least equal to the functionality of CM achieved from mitotic inactivated feeder cells in t-flasks. Three bioreactor cultivatons had been done under same conditions. As well as the cultures showed similar behaviour during the process the harvested medium had the same quality concerning the functionality.

Comparable with inactivated feeder cells the mouse embryonic fibroblasts in the bioreactor remain in a stationary phase. Besides glucose the feeder cells utlilize pyruvate as carbon source, like early human embryonic cells do. As shown by a test of metabolite depleted medium, the small amounts of built metabolites does not affect the functionality of the CM.

A future goal is the establishment of a process with human feeder cells.