# ZENTRUM ANGEWANDTE CHEMIE

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# Optimization of in-vitro expansion of mesenchymal stem cells isolated from human umbilical cord


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***Introduction:*** *Acute demand of human mesenchymal stem cells (hMSC) exists in the areas of regenerative medicine and cell based therapies. The extraction of hMSC from bone marrow, the most commonly used source for these stem cells, is often less effective because of the low frequency of hMSC (0,01-0,001%) in marrow aspirates. Investigations over the last years show that hMSC can be successfully isolated also from extra-embryonic tissues such as placenta, amniotic membrane or umbilical cord (UC), where the frequency of MSC is substantially higher. Our main goal is to optimize in-vitro culture conditions for extensive expansion of hMSC obtained from perivascular tissue of umbilical cord, known as human umbilical cord perivascular cells (HUCPV-cells*) or whole UC, so called human umbilical cord matrix cells (HUCM-cells).*

***Materials and methods:*** *At first 1x10$^5$ HUCPV-cells were seeded in culture flasks at the density of 4000 cells/cm$^2$ and grown in four slightly different αMEM (Table 1.) over 6 passages. All media were supplemented with 10% human serum (HS) and 50 µg/ml gentamicin. In the second step 1x10$^5$ cells were first seeded at 7, subsequently at 3 different densities varying from 62.5 to 4000 cells/cm$^2$ (Table 2.) and propagate in αMEM containing 2 mM L-glutamine over one passage only. Cell proliferative capacity, vitality, viability and consumption of crucial nutrients (glucose, L-glutamine and essential amino acids) were monitored by microscopic (trypan blue exclusion test, colony-forming unit-fibroblast (CFU-F) test), fluorescencespectroscopic (CellTiter-Blue® Cell Viability Assay, CytoTox-One™ Homogeneous Membrane Integrity Assay, Apo-One® Homogeneous Caspase-3/7 Assay, all from Promega) and HPLC methods. The comparative analysis of cell surface epitopes (CD90, CD105, CD73, CD44, CD34, CD45 and HLA-I) were performed by using flow cytometry.*

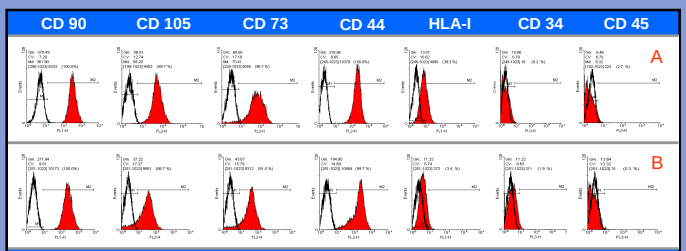
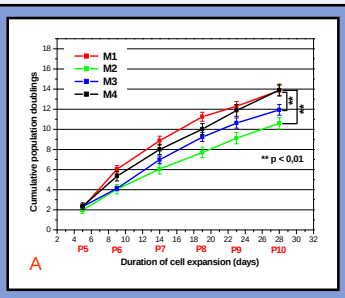



*Figure 1. Immunophenotypic characteristics of HUCPV- (A) and HUCM-cells (B).*



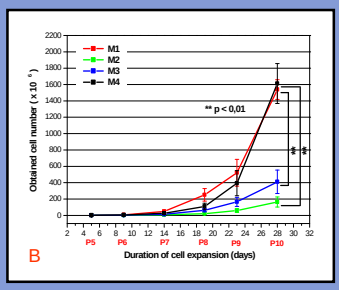
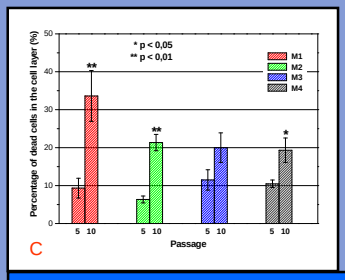




***Table 1.***
***Differences in the composition of used αMEM***

| Medium | Difference |
|---|---|
| M1 | - Reference medium - |
| M2 | Glutamax instead of L-glutamine |
| M3 | Additionally buffered with HEPES |
| M4 | Extra supplemented with osteogenic factors (0,2 mM L-ascorbate-2-phosphate, 5 mM β-glycerophosphate, 10 nm dexamethasone) |

***Table 2.*** ***Cell seeding densities***
*Total number of seeded cells: 1 x 10$^5$*

| Cell density (cells/cm$^2$) | Cell seeding area (cm$^2$) |
|---|---|
| 4000 | 25 |
| 2000 | 50 |
| 1000 | 100 |
| 500 | 200 |
| 250 | 400 |
| 125 | 800 |
| 63 | 1600 |



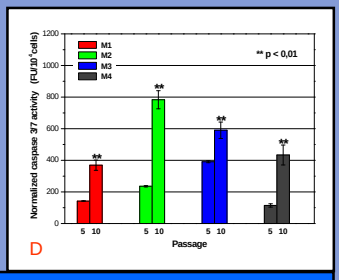




*Figure 3. Cell population doublings (A), obtained cell number (B) and changes in the cell viability (C,D) during HUCPV-cell expansion over 6 passages in four different supplemented αMEM (Tab. 1.).*

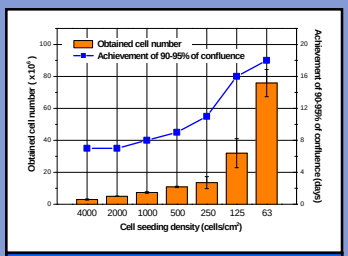



*Figure 3. Relation between initial cell seeding density, number of obtained cells within one passage and time of achievement of 90-95% of confluence. In each case 1 x 10$^5$ HUCPV-cells were initially seeded.*

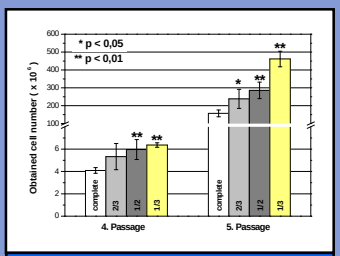
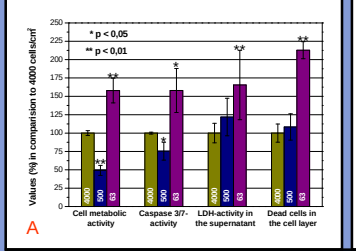



*Figure 4. Influence of complete and partial (2/3, 1/2, 1/3) medium change on proliferative activity of HUCPV-cells. 1 x 10$^5$ cells were seeded at a density of 500 cells/cm$^2$ and expanded over two passages.*



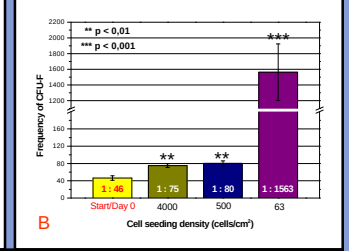




*Figure 5. Influence of cell seeding density on cell viability (A) and frequency of colony-forming units-fibroblasts (CFU-F) (B). HUCM-cells were seeded at three different densities and cultivated over one passage. Parameters of cell viability were determined at the moment of ~ 90% of confluence. Parameters were first normalized to the total cell number and subsequently compared with values determined in cultures with an initial cell seeding density of 4000 cells/cm². For the CFU-F test 50 cells/cm² were seeded into 75-cm² flasks at the beginning and at the end of each cell expansion procedure. Produced colonies, comprising more then 20 cells, were counted on day 10.*

***Results:*** *HUCPV- as well as HUCM-cells are highly positive for typical stem cell surface markers such as CD73, CD90, CD105 and CD44. Both exhibit remarkable proliferative activity particularly supported by αMEM containing 2 mM L-glutamine or αMEM additionally supplemented with osteogenic agents (Fig. 2. A,B). In any case cell long-term expansion is connected with deprivation of their proliferative activity and viability (Fig. 2. C,D). If initial cell seeding density of 4000 cells/cm² is used, cells must be enzymatically dissociated and splitted every 4-5 days. This process is associated with regular destruction of cell-cell and cell-matrix contacts and therefore with additional cell damage or even loss. In order to reduce the number of cell enzymatic dissociations and increase the cell number gained within one passage, we decreased the cell seeding density (Tab. 2.). Initial cell seeding at the density of 500 cells/cm² in place of 4000 cells/cm² allows to get approx. 3,5 times more cells within one passage without the intrinsic loss of cell viability (Fig. 3., 5.). In this case cell growth is additionally facilitated by partial medium change (Fig. 4.). Using this cultivation method, the routine monitoring of L-glutamine, glucose and lactate concentration in the conditioned medium and culture supernatant is mandatory. In present case regular replacement of 1/3 or 1/2 of cell culture supernatant with fresh medium was possible without limitations in the supply of crucial nutrients over two passages only (data not shown). Lower cell seeding densities than 500 cells/cm² is associated with non-uniform colonization of the cultivation area as well as with essential loss of cell viability and proliferative capacity (Fig. 5.).*

***Conclusions:*** *Minor differences in the culture media supplementation, the initial density of cell seeding or the time period of cell progeny have a substantial impact on the proliferative capacity and viability of HUCPV- and HUCM-cells. Cell seeding at a density of 500 cells/cm² on a larger cultivation area allows to avoid frequent enzymatic disruption of cell-cell contacts, reduces the risk of contaminations and therefore displays a better cell expansion strategy. However further optimization of methods for the extensive expansion of these MSC, such as cell cultivation under conditions of physiological hypoxia, must be accomplished.*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