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Viability of the Human Adenocarcinoma Cell Line Caco-2: Influence of Cryoprotectant, Freezing Rate, and Storage Temperature

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Abstract

The freezing process as well as cryostorage may induce molecular and cellular changes due to osmotic stress. Currently, standard protocols for cryopreservation of mammalian cells recommend slow freezing and rapid thawing to avoid intracellular ice crystal formation and osmotic damage. As an appropriate freezing process should guarantee adequate cell viability, we evaluated the impact of two cryoprotectants, a commercially available cryopreservation medium, two freezing rates, as well as two storage temperatures on the viability of Caco-2 cells. The freezing parameters were optimized by carefully determining vitality, cell count, proliferation, and functional differentiation of the cells. Though at least 90% of the cells were viable after one freezing/thawing cycle, adequate recovery of proliferation and differentiation is obtained not until 10 days post thawing. Interestingly, addition of 10% PEG 200 as cryoprotectant yielded best results. All in all, our results should give valuable advice for the optimization of the cryopreservation protocol of individual cell lines.

Keywords

Caco-2 • Cryopreservation • Freezing rate • Cryoprotectant • Viability

Introduction

During the last years human cell lines have become an accepted tool in pharmaceutics to investigate uptake, bioavailability, and metabolism of drugs. Among a lot of different cell lines, the human adenocarcinoma derived Caco-2 cells, which form a columnar absorptive epithelium with tight junctions similar to the human small intestine, were recognized as a valuable tool in the field of oral absorption [1]. Later on, the Caco-2 cell line was recommended by the FDA as an integral component of the Biopharmaceutics Classification System (BCS) [2]. Consequently, routine use of Caco-2 cells for screening biopharmaceutical parameters of new drug candidates in pharmaceutical industry requires reproducible characteristics necessitating a vital stock of cells and consequently an optimum freezing protocol.

Current standard protocols for cryopreservation of mammalian cells suggest to freeze the cells in 1 ml cryovials containing culture medium, 5–20 % (v/v) fetal bovine serum, and dimethyl sulfoxide (Me_2SO, usually 10% (v/v)) as a cryoprotectant [3]. Furthermore, slow freezing and rapid thawing rates are applied to minimize intracellular ice formation and osmotic damage [4]. For long-term preservation cells are stored in liquid nitrogen at temperatures between −130°C and −196°C.

However, the freezing process induces cellular and molecular changes caused by osmotic stress, which might reduce cell viability and negatively influence cellular adhesion, proliferation, and differentiation [5]. In an attempt to guarantee high cell vitality as well as proper adhesion and differentiation of the thawed cells, many alternative freezing protocols are described in the literature focusing on an optimized cryopreservation process for different cell types [6–7].

The aim of this work was to investigate the influence of various freezing parameters on vitality, adhesion and differentiation of Caco-2 cells and, finally, to establish an optimum protocol for the cryopreservation of this cell line. Additionally, this study might give valuable advice for the optimization of freezing protocols for other cell types.

Experimental

Cell Culture

Caco-2 cells (DSMZ, Braunschweig, Germany) of the passages 20–29 were grown to confluency in RPMI-1640 cell culture medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FCS; Gibco, Lofer, Austria) in a humidified 5% CO_2 / 95% air atmosphere at 37°C using 75 cm^2 tissue culture flasks (Nunc Easyflask 75 Filt, Nalgene Nunc International, Rochester, NY, USA) and subcultured by animal origin free TrypLE Select (Gibco).

Cryopreservation protocols

The cryopreservation medium consisted of equal volumes of RPMI-1640 and FCS. This optimum amount of FCS in RPMI-1640 for cryopreservation of Caco-2 cells was investigated in a former test series (data not shown). To enhance the performance, the cryopreservation medium was supplemented with 5% Me_2SO, 10% Me_2SO or 10% polyethyleneglycol 200 (PEG 200) as a cryoprotectant. Moreover, the commercially

available cryopreservation medium CryoStor™ CS10 (VWR International, Darmstadt, Germany) containing 10% Me_2SO was applied and evaluated.

After determining the viability of the splitted cells (protocol below), the Caco-2 cells were frozen at a density of $2.5x10^6$ living cells/ml in polypropylene cryovials (Nalgene Nunc International). For this purpose, the cell culture medium was replaced by the cryopreservation after a centrifugation step (1,000 rpm / 5 minutes). Following an equilibration time of 30 minutes at +4°C, the cells were frozen using two different freezing rates (Planer plc Kryo 560-16, Planer, Middlesex, United Kingdom). The freezing rates investigated were −1°C/min and −10°C/min and the end temperature of the controlled freezing was set to −80°C or −180°C. To investigate optimum cryopreservation conditions, the samples were stored either at −80°C or in the vapor phase of liquid nitrogen for a minimum of three days. Additionally, cells were stored at −80°C immediately after the equilibration time without any control of the freezing rate. In the results part, the respective cryopreservation protocol of the individual samples is given in square brackets as follows: [freezing rate per minute / end temperature of controlled freezing / cryostorage temperature].

Immediately after thawing the samples at 37°C for 5 min the cryopreservation medium was replaced by RPMI-1640 medium and the membrane integrity test was performed as described below to assess the vitality of the cells after one freezing/thawing cycle.

Viability studies

The viability of the cells was determined using a cell membrane integrity assay as described in the literature [8]. In brief, equal volumes of the cell suspension and a solution containing 19.2 µM fluorescein diacetate (FDA; Sigma) and 25 µM ethidium bromide (EB; Sigma) in RPMI-1640 medium were mixed followed by counting the number of living (green) and dead (red) cells using fluorescence microscopy at 450–490 nm/ 515-555 nm (green) and 590 nm (red), respectively, and a Fuchs-Rosenthal haemocytometer (Brand, Wertheim, Germany).

Proliferation and differentiation studies

To investigate the impact of the different freezing parameters on proliferation and differentiation of the cells after cryopreservation Caco-2 cells were seeded after thawing into the wells of a 96-well microplate (Sarstedt, Nürnbrecht-Rommelsdorf, Germany) at a density of 31,250 living cells per cm^2 and cultured for 3, 7 or 10 days. As a reference, subcultured Caco-2 cells of the same passage number but without any freezing were seeded at the same density.

The proliferation rate was determined using the BrdU cell proliferation ELISA test kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. DNA-Incorporation of BrdU was quantified using a microplate reader (Spectra Max 340, Molecular devices, Sunnyvale, CA, USA) at 450nm.

Functional differentiation of the cells was investigated via the activity of the brush border hydrolase aminopeptidase N [9]. To assess the enzyme activity of the cell layer, the supernatant culture medium was removed and 150 µl substrate solution containing 1.5 mM L-alanine-7-amido-4-methylcoumarine trifluoroacetate salt in isotone 20 mM Hepes/NaOH

buffer pH 7.4 were added. Following incubation at 37°C for 1 h in the dark, 100 µl of the supernatant were transferred into a 96 well microplate. The converted substrate was quantified in a microplate fluorimeter (FL-500, Bio-Tek, Winooski, Vermont, USA) at 360nm/460nm using substrate without cells as a blank. The activity of aminopeptidase N was calculated from a standard curve of 7-amino-4-methylcoumarine in 20 mM Hepes/NaOH buffer pH 7.4. One unit is defined as the amount of enzyme that hydrolyses 1.0 µmol L-alanine-7-amido-4-methylcoumarin trifluoroacetate salt per minute at standardized conditions as described above.

To determine the number of adherent cells the DNA content of the cells was estimated by the CyQuant™ test kit (Molecular Probes, Eugene, OR, USA) pursuing a modified protocol. After removal of the supernatant the cells were lysed by incubation with 50 µl of a mixture of 25 µl 20mM Hepes/NaOH buffer pH 7.0 and 25 µl CelLytic™-M Cell Lysis Reagent (Molecular Probes) for 15 min at room temperature. The DNA of the lysed cells was stained by adding 150 µl dyeing solution representing a mixture of 0.5 µl fluorescent dye with 7.5 µl buffer and 142.5 µl water, according to the manufacturer's instructions. After incubation for 20 min in the dark the fluorescence intensity was determined at 485/530nm in a fluorescence microplate reader (FL-500, Bio-Tek).

All assays were performed at least in triplicate and repeated three times to assure statistical relevance.

Results and Discussion

Viability studies

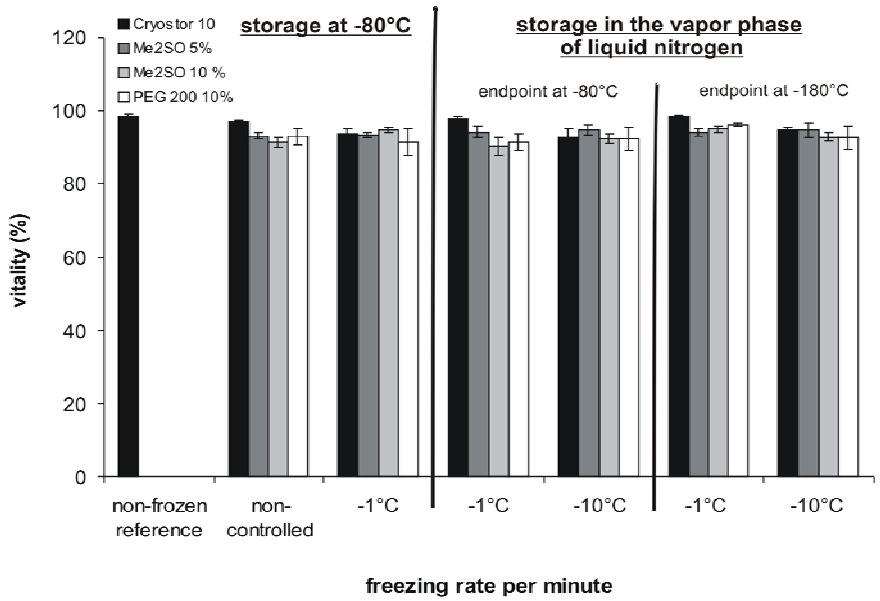

Fig. 1. Vitality of Caco-2 cells after one freezing/thawing cycle. Cryoprotectants: Me_2SO 5%, Me_2SO 10% and PEG 200 10% (all v/v) in RPMI-1640 and FCS (1+1) or CryoStor CS10 (containing 10% Me_2SO); freezing rates: −1°C, −10°C, non-controlled freezing (storage at −80°C immediately after equilibration time); endpoint of controlled freezing: −80°C or −180°C; cryostorage temperature: −80°C or in the vapor phase of liquid nitrogen (−130°C to −196°C); (n=3, mean ± SD).

To elucidate the impact of different freezing parameters the number of viable cells was calculated after a cryopreservation step using a cell membrane integrity assay and compared to a non-frozen reference.

As shown in Fig.1, the membrane integrity test after one freezing/thawing cycle revealed that there is no pronounced influence of the freezing procedure as well as the cryoprotectant on the vitality of the cells amounting to at least 90%. 98.38% ± 0.74 of the cells of the non-frozen reference were alive which is comparable to that of cells frozen in CryoStor CS10 [−1°C / −180°C / −130°C to −196°C] (98.3% ± 0.28). The same cryopreservation procedure, but applying controlled freezing down to −80°C and CryoStor CS 10 as cryopreservation medium (97.85% ± 0.49), as well as storing the cells at −80°C without any controlled freezing rate (96.95% ± 0.38) yielded similar results. Using PEG 200 as cryoprotectant 96.11% ± 0.49 of the cells were viable after thawing. In case of Me_2SO as cryoprotectant the maximum vitality was 94%. All in all, CryoStor CS10 maintains the vitality of Caco-2 cells at a high level and seems to be a proper cryopreservation medium. Further, a freezing rate of -1°C and the storage in the vapor phase of liquid nitrogen lead to a slightly higher vitality as compared to other investigated freezing rates and -80°C as cryostorage temperature. Interestingly, non-controlled freezing followed by storage at −80°C resulted in slightly higher vitality as compared to the controlled procedure at a rate of −1°C/min. When applying 10% Me_2SO for cryoprotection a freezing rate of -1°C/min (94.73% ± 0.81 [−1°C / −80°C / −80°C] or 94.92% ± 0.74 [−1°C / −180°C / −130°C to −196°C]) seems to be preferable as compared to −10°C/min (92.9% ± 1.1 [−10°C / −180°C / −130°C to −196°C]), independent of the cryostorage temperature.

In case of using only 5% Me_2SO, the freezing rate seemed to have no major impact on the vitality yielding about 94% in all samples.

Proliferation and differentiation studies

Evaluation of the proliferation rate of Caco-2 cells after one freezing/thawing cycle revealed that the proliferative activity 3 days post-thawing is reduced to about 50% as compared to non-frozen cells (data not shown), especially of cells that were frozen with 10% Me_2SO as a cryoprotectant (0.27 ± 0.07 AU (absorbance units) as compared to 0.55 ± 0.00 AU of the reference). After 7 days in culture the proliferative activity of nearly all cells was around 0.7 AU, independent of the former cryopreservation parameters, as compared to 1.02 ± 0.15 AU of the control (Fig. 2). Interestingly, the proliferation rate of cells frozen using 5% Me_2SO as cryoprotectant was about 15% lower.

Ten days post-thawing the proliferation rate reached that of the non-treated cells (1.07 ± 0.16 AU; Fig. 2), indicating that the metabolism of the cells had nearly completely recovered. However, in case of CryoStor CS 10 as a cryoprotectant and a freezing rate of −10°C the proliferative activity of cells was lower, yielding 0.85 ± 0.03 AU [−10°C / −180°C / −130°C to −196°C] or 0.84 ± 0.03 AU [−10°C / −80°C / −130°C to −196°C], respectively. Furthermore, applying 10% Me_2SO as cryoprotectant and using a freezing rate of −10°C resulted in a slightly lower proliferative activity (0.88 ± 0.01 AU) of the cells than the reference. Using only 5% Me_2SO as cryoprotectant caused a reduction of about 25% as compared to the control. PEG 200 was the only cryoprotectant that maintained a similar proliferative activity of the cells as compared to the controls after 10 days in culture (0.93 ± 0.01 - 1.15 ± 0.06 AU), independent of the cryopreservation parameters applied.

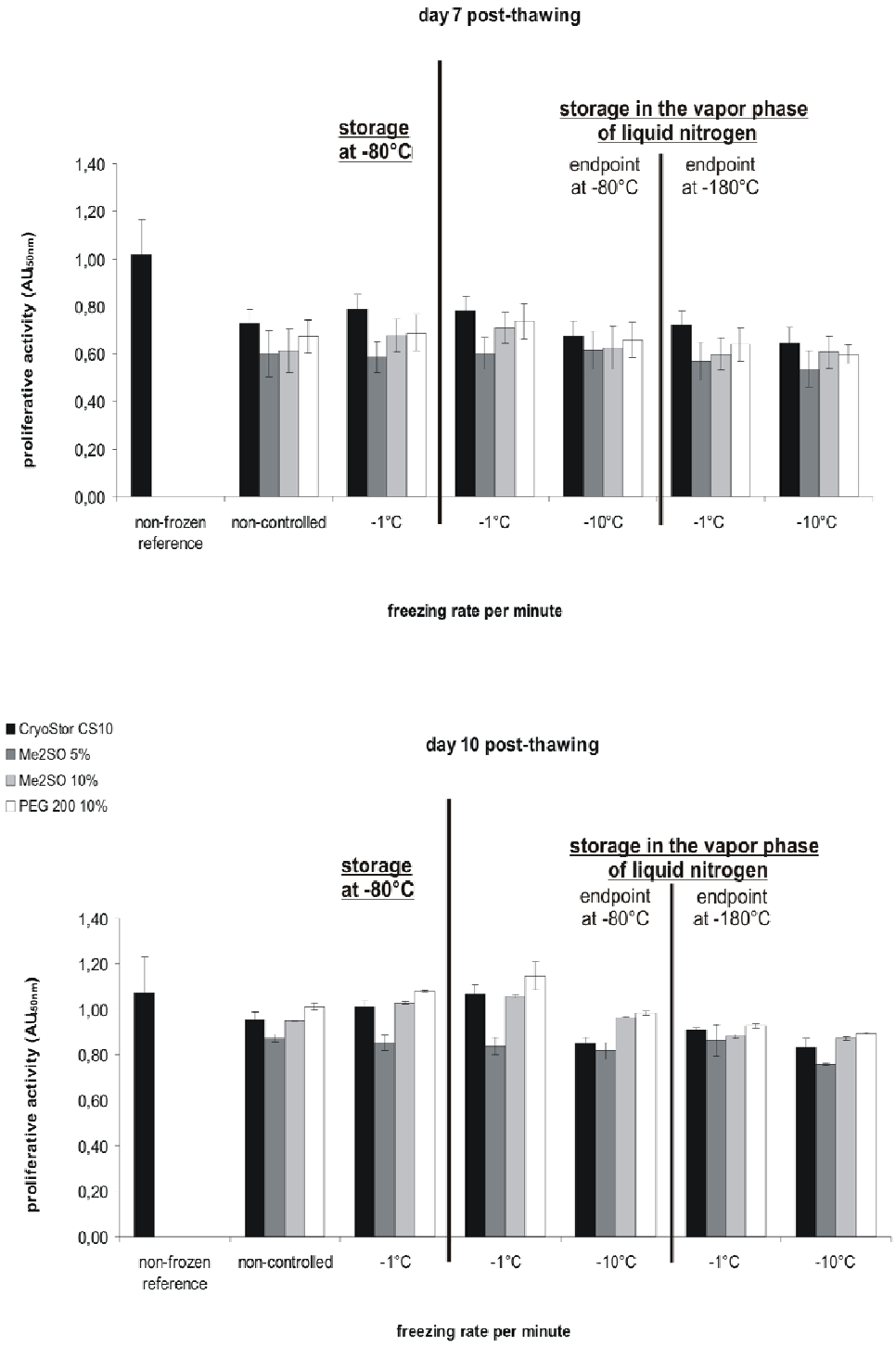

Fig. 2. Proliferative activity of Caco-2 cells after one freezing/thawing cycle on day 7 and day 10 post-thawing (n=3, mean ± SD).

Finally, the degree of the functional cell differentiation was evaluated by determining the activity of the brush border hydrolase aminopeptidase N (APN). Whereas 3 days post-seeding even the non-frozen reference cells exhibited a very low initial differentiation (11.50 ± 0.10 nU/100,000 cells), after 7 days in culture the activity of the APN was increased by more than 50% (18.44 ± 0.73 nU/100,000 cells). Interestingly, the enzyme activity of frozen cells reached only about 70% of the reference on day 7 post-thawing. However, 10 days post-thawing the impact of the cryopreservation procedure on cell differentiation became more apparent. As shown in table 1, the differentiation of cells stored at −80°C was considerably higher than that of cells stored in the vapor phase of liquid Nitrogen. Without freezing the APN activity of the controls amounted to 23.12 ± 1.35 nU/100,000 cells. Similar results were observed only upon storage at −80°C without controlled freezing using PEG 200 as cryoprotectant. When the cells were stored in the vapor phase of liquid nitrogen, again PEG 200 yielded the best results with a differentiation rate of about 77% as compared to the reference, independent of the endpoint of controlled freezing. Using Me_2SO for cryoprotection the enzyme expression of the Caco-2 cells was only 52–59% at the most as compared to the non-frozen cells.

Tab. 1. Influence of the cryopreservation procedure on the activity of aminopeptidase N (APN) 10 days post-thawing (n=3, mean ± SD).

		Aminopeptidase N day 10 post-thawing (nU/100,000 cells)					
storage		**-80°C**		**vapor phase of liquid nitrogen**			
freezing rate *(°C/min)*		**non-controlled**	**−1**	**−1**	**−10**	**−1**	**−10**
freezing applied till				**−80 °C**		**−180 °C**	
cryoprotectant	CryoStor CS10	20.14 ± 0.33	19.15 ± 0.18	15.57 ± 0.35	16.74 ± 1.08	17.00 ± 1.19	17.59 ± 0.11
	Me_2SO 5%	16.94 ± 0.46	17.34 ± 0.44	13.66 ± 0.64	13.39 ± 0.84	11.87 ± 0.31	11.88 ± 0.24
	Me_2SO 10%	19.80 ± 0.12	18.10 ± 0.74	13.46 ± 0.79	12.10 ± 0.57	11.90 ± 0.64	13.55 ± 0.52
	PEG 200 10%	22.76 ± 0.20	20.48 ± 1.00	17.53 ± 0.23	17.56 ± 0.91	17.94 ± 0.17	16.08 ± 0.48

Conclusions

All in all, about 90% of Caco-2 cells are viable after one freezing/thawing cycle, almost independent of the cryoprotectant and the cryopreservation procedure used. However, full proliferative activity of the cells is recovered not until 10 days in culture. By that time the ability of the Caco-2 cells to express functional APN was still restricted. Interestingly, PEG 200 might be a suitable cryoprotectant for the Caco-2 cells since freezing in presence of 10% PEG 200 provoked sufficient proliferative activity and highest differentiation on day 10 post-thawing as compared to all other samples. Moreover, the commercially available cryopreservation medium CryoStor CS10 proved to be useful due to full recovery of the

cells, as shown by Baust et.al. [10]. In contrast, the commonly used cryoprotectant Me_2SO yielded only moderate results with respect to the proliferative activity and the differentiation rate 10 days after thawing. Interestingly, storing the cells at −80°C immediately after equilibration led to adequate recovery of the cells, although a freezing rate of −1°C seems to be preferable for Caco-2 cells. Nevertheless, it should be considered that this study reports about short-time storage and further investigations are necessary for long-time preservation. Moreover, a rather rapid equalization of the observed differences in enzyme activity might be conceivable after subcultivating thawed confluent cell layers. Although these studies prove that Caco-2 cells represent a very robust cell line, even in this case careful optimization of the freezing procedure is inevitable upon routine use in biopharmaceutical screening.

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Authors’ Statement

Competing Interests

The authors declare no conflict of interest.

References

[1] Hidalgo IJ, Raub TJ, Borchardt RT.
Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability.
Gastroenterology. 1989; 96: 736–749.
PMid:2914637

[2] Kim JS, Mitchell S, Kijek P, Tsume Y, Hilfinger J, Amidon GL.
The suitability of an in situ perfusion model for permeability determinations: Utility for BCS Class I biowaiver requests.
Mol Pharm. 2006; 3: 686–694.
doi:10.1021/mp060042f

[3] McLellan MR, Day JG.
Cryopreservation and freeze-drying protocols. Introduction.
Methods Mol Biol. 1995; 38: 1–5.
doi:10.1006/cryo.1996.0051

[4] Mazur P.
Freezing of living cells: Mechanisms and implications.
Am J Physiol. 1984; 247: C125–C142.
PMid:6383068

[5] Woods EJ, Benson JD, Agca Y, Crister JK.
Fundamental cryobiology of reproductive cells and tissues.
Cryobiology. 2004; 48: 146–156.
doi:10.1016/j.cryobiol.2004.03.002

[6] Ji L, de Pablo JJ, Palecek SP.
Cryopreservation of adherent human embryonic stem cells.
Biotechnol Bioeng. 2004; 88: 299–312.
doi:10.1002/bit.20243

[7] Leibo SP, Farrant J, Mazur P, Hanna MG, Smith LH.
Effects of freezing on marrow stem cell suspensions: Interactions of cooling and warming rates in the presence of PVP, sucrose, or glycerol.
Cryobiology. 1970; 6: 315–332.
doi:10.1016/S0011-2240(70)80086-4

[8] Dankberg F, Persidsky MD.
A test of granulocyte membrane integrity and phagocytic function.
Cryobiology. 1976; 13: 430–432.
doi:10.1016/0011-2240(76)90098-5

[9] Piana C, Güll I, Gerbes S, Gerdes R, Mills C, Samitier J, Wirth M, Gabor F.
Influence of surface modification on vitality and differentiation of Caco-2 cells.
Differentiation. 2007; 75: 308–317
doi:10.1111/j.1432-0436.2006.00141.x

[10] Baust JM, Van Buskirk, Baust JG.
Cell viability improves following inhibition of cryopreservation-induced apoptosis.
In Vitro Cell Dev Biol Anim. 2000; 36: 262–270.
doi:10.1290/1071-2690(2000)036<0262:CVIFIO>2.0.CO;2