Hyperosmolality in CHO Culture: Effects on cellular behavior and morphology

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We have no known conflict of interest to disclose.

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ABSTRACT

Exposure of Chinese hamster ovary cells (CHO) to highly concentrated feed solution during fed-batch cultivation is known to result in an unphysiological osmolality increase (>300 mOsm/kg), affecting cell physiology and morphology. Extending previous observation on osmotic adaptation, the present study investigates for the first time potential effects of hyperosmolality on CHO cells on both population and single-cell level.

We intentionally exposed CHO cells to hyperosmolality of up to 545 mOsm/kg during fed-batch cultivation. Contrarily to an expected osmosis effect promoting cell shrinkage, hyperosmolality-exposed CHO cells showed a nearly triplicated volume accompanied by ablation of proliferation. On the molecular level, we observed a strong hyperosmolality-dependent increase in mitochondrial activity in CHO cells compared to control. The companion article “Hyperosmolality in CHO Culture: Effects on Proteome” provides a proteome-based insight into the effects of hyperosmolality on mitochondria. In contrast to mitochondrial activity, hyperosmolality-dependent proliferation arrest of CHO cells was not accompanied by DNA accumulation or caspase-3/7-mediated apoptosis. Notably, we demonstrate for the first time a formation of up to eight multiple, small nuclei in single hyperosmolality-stressed CHO cells.

The here presented observations reveal unknown hyperosmolality-dependent morphological changes and support existing data on the osmotic response in mammalian cells.

Keywords**: CHO, fed-batch, hyperosmolality, mitochondria, cell size, cell morphology**

INTRODUCTION

Up to date, the majority of biopharmaceuticals, such as antibodies or their fragments, growth factors, cytokines, and Fc-fusion proteins are produced in mammalian cell lines. The ability of these cells to promote proper protein folding, replicate human-like posttranslational modifications, coupled with their high cell-specific productivity and ability to grow in suspension makes these cell lines highly attractive (Fischer et al., 2015, Kim et al., 2011). CHO cell-based protein production systems are by far the most commonly used in industry, accounting for 84% of the monoclonal antibody (mAb) products approved up to 2018 (Walsh, 2018).

CHO production processes are usually performed in a fed-batch mode using repeated bolus or continuous feeding, which leads to increased cell density and product titers. The high nutrient content of the medium concentrates being used results in a substantial increase in medium osmolality, which is known to affect cellular behavior and productivity (Bibila & Robinson, 1995, J.S. & G.M.Lee, 1999). Several studies have investigated the effects of osmolality change on the growth and productivity of CHO cells (Kiehl et al., 2011, Pan et al., 2017, Shen et al., 2010). In most cases an increased cell-specific productivity (Nasseri et al., 2014, Qin et al., 2019, Takagi et al., 2000, Wang et al., 2012) with concomitant depressed cell growth (Kim & Lee, 2002) has been found, but also surprising results like an increase in cell size under hyperosmolality have been reported. This seems to be inconsistent with classical osmosis that should result in cells' shrinking by loss of cytoplasmic water, as observed in bacteria (Dai & Zhu, 2018) and yeast (Pratt et al., 2003).

To investigate this surprising size increase and to obtain a more holistic overview of the effect of hyperosmolality on CHO cells, we intentionally "overfed" the antibody-producing CHO DP-12 cells with specifically tailored industrially relevant CHO feed and studied the effects of osmolality increase on a populational and single-cell level.

1. MATERIALS AND METHODS

1.1 Cell culture maintenance

The influence of high osmolality was studied primarily in suspension-adapted CHO DP-12 clone#1934 (ATCC CRL-12445) cells. It co-expressed the variable light and heavy chains of the murine 6G4.2.5 monoclonal antibody (ATCC-HB-11722) which inhibits binding of interleukin 8 (IL-8) to human neutrophil. Gene integration was stabilized by DHFR/MTX system. 200 nM methotrexate (MTX, Sigma-Aldrich, St. Louis, MI, USA) was present during pre-culture but not during the main cultivation.

CHO DP-12 cells were cultivated in chemically defined medium TCX6D (Xell AG, Bielefeld, Germany) supplemented with 8 mM glutamine.

For each experimental cultivation a new vial containing 1×10^7 cells was thawed, washed in PBS once, and resuspended in ca. 15 ml of growth medium. All vials originated from the same master cell bank. Pre-culture was maintained in vented conical flasks (50 ml TubeSpin, TPP Techno Plastic Products AG, Trasadingen, Switzerland) and routinely passaged every three days. Cell concentration, viability, and cell diameter were determined with a Cedex AS20 system (Innovatis-Roche AG, Bielefeld, Germany). Each measurement was based on 20 valid images. The parameters of about 200 to up to 8000 cells were measured per sample. Cells were seeded with a viable cell density (VCD) of 3×10^5 cells/ml.

Incubation parameters in an orbital shaker incubator (Mytron GmbH, Heiligenstadt, Germany) were maintained at 37 °C with a relative CO_2 concentration of 5%, 80% humidity, and with shaker agitation of 185 rpm.

1.2 Fed-batch cultivation

Cells for three to four biological replicates for each of two conditions ("feed" condition and "control" condition) were seeded with a VCD of 3×10^5 cells/ml in a 125 mL polycarbonate un-baffled shake flask (Corning Life Sciences B.V., Amsterdam, Netherlands) in an initial volume of 40 ml and cultivated as described before.

For feed-condition shakers, CHO Basic Feed (Xell AG, Bielefeld, Germany) was supplemented with 404 mM glucose, 70 mM glutamine, and 27 mM asparagine. 72 h post-seeding, 6 ml of supplemented feed were added to the feed-condition shakers for the first time. This procedure was repeated four times in total, roughly once every 24 h. To cancel out any dilution effects, 6 ml of the Gln-supplemented growth medium was added to control-condition shakers at the same time points.

The product (anti-IL-8 antibody) concentration was estimated using Protein A HPLC after finishing the cultivation. Daily glucose and lactate measurements were performed using cell-free supernatant on a Biosen C-line Clinic (GEMAR GmbH, Celle, Germany). Osmolality was measured daily using an Osmomat Auto (Gonotec GmbH, Berlin, Germany).

1.3 Cell cycle analysis

Cell cycle distribution was analyzed on BioRad S3E (BioRad Laboratories Inc., Hercules, CA, USA) Data was evaluated with FlowJo™ v10.6.2 (FlowJo, now BD Bioscience, Ashland, OR, USA) software. 2×10^6 cells were harvested at four designated time points (day two, four, five, and seven), washed twice with ice-cold phosphate-buffered saline (PBS), permeabilized with ice-cold 70% ethanol, and stored at – 20 C at least overnight. Before measurement, cells were washed twice with 0.1% saponin in PBS, followed by incubation for 45 min in the dark at room temperature with 20 µg/ml/10^6 cells propidium iodide (PI) and 40 µg/ml/10^6 cells RNAse A. After

the incubation, cells were stored on ice and vortexed prior to each measurement and strained through nylon-mesh CellTrics filters with 30 µm pore size (Sysmex, Kōbe, Hyōgo, Japan) directly into the measuring tube. 180,000 events per sample were collected at a low acquisition rate (200 to 300 cells/s). The propidium iodide fluorescence is proportional to the DNA content of the cell and was detected in the FL2 channel.

1.4 Mitochondria activity assessment

MitoTracker® Red CMXRos (Cell Signalling Technology Inc., Danvers, MA, USA) reagent was used to characterize mitochondrial mass and activity in the cells using flow cytometry. The dye is sensitive to mitochondrial membrane potential and can be used to access the functionality of mitochondria (Puleston, 2015). It diffuses passively through the cell membrane and accumulates in active mitochondria.

$1{,}5\times10^6$ cells were sampled from each shaker at three designated time points (day two, six, and eight), centrifuged at $200\times g$ and resuspended in 100 nM MitoTracker® Red CMXRos reagent diluted in growth medium to a concentration of 2×10^6 cells/ml. The samples were incubated at 37 °C on an orbital shaker (185 rpm) for 45 min in the dark. Nuclei were counterstained with 1 µM 4′,6-Diamidin-2-phenylindol (DAPI, 1:1000 dilution of 1 mM DAPI stock, Sigma, now Merck, Darmstadt, Germany) for 10 min at 37° C. Following the counterstaining step, cells were strained through nylon-mesh CellTrics filters with 30 µm pore size (Sysmex, Kōbe, Hyōgo, Japan) directly into flow cytometry tube.

Measurement was performed on Navios EX flow cytometer (Beckman Coulter, Brea, CA, USA). Dead cells were gated out based on DAPI signal and intensities of

MitoTracker stained cell populations were compared at an emission maximum of 561 nm.

1.5 Apoptosis assay

An apoptotic signal was detected by assaying intracellular Caspase 3/7 activation using CellEvent® Caspase-3/7 Green Detection Reagent (Thermo Fisher Scientific Inc., Carlsbad, CA, USA), which is not toxic to the cells. It is an Asp-Glu-Val-Asp (DEVD) peptide, conjugated to a nucleic acid binding dye. This conjugate is cell-permeable but intrinsically non-fluorescent unless it is bound to the DNA. The DEVD peptide inhibits the ability of the dye to bind DNA and must be cleaved away by an activated caspase 3 or caspase 7 protease for fluorescence to occur. The bright green signal was detected at 514 nm on Navios EX flow cytometer.

$1{,}5\times10^6$ cells were collected at the previously indicated time points, centrifuged at $200\times g$ for 5 min. and resuspended in 300 µl of growth medium with the same osmolality. The Caspase 3/7 reagent was added in a concentration of 5 µM to the positive samples, the same volume of the growth medium was added as vehicle control. Both positive and negative samples were incubated for 30 min at 37° C, 5% CO_2 and humidity of 80% without shaking.

After the incubation cells were counterstained with DAPI at 1 µM and incubated for 10 min as before. Before measurement, the cells were diluted to a final concentration of 2×10^6 cells/ml and strained through nylon-mesh CellTrics filters with 30 µm pore size (Sysmex, Kōbe, Hyōgo, Japan) directly into flow cytometry tube.

1.6 Confocal microscopy and immunocytochemistry (ICC)

Samples for all confocal microscopy analyses were taken at the same time points as those for proteome and live-stains: at days two, six, and eight. Only one biological

replicate per feed and control conditions was examined. The mitochondria live stain MitoTracker® Red CMXRos and Caspase 3/7 green detection reagent can be used for an end-point assay if a sample is fixed immediately after incubation.

For confocal microscopy, $0{,}5\times10^6$ cells were sampled from one of the shakers per condition. The sample was stained with 200 nM MitoTracker® Red CMXRos for 45 min at 37 °C on an orbital shaker (185 rpm) in the dark. To omit a bright red background, the sample was washed three times with PBS and then fixed in 250 µl of 100% ice-cold methanol for 15 min at -20 °C according to manufacturer instructions.

Samples stained for apoptosis detection via Caspase 3/7 activation (incubated for 30 min at 37 °C, 5 µM Caspase 3/7 green detection reagent) were fixed with 4% polymeric formaldehyde (PFA, Carl Roth GmbH, Karlsruhe, Germany) for 20 min at room temperature. 100 µl of each readily fixed sample were spun down onto ibidi 8 - chamber slides with ibidi-treat surface (ibidi GmbH, Graefelfing, Germany) at $800\times g$.

Samples with stained mitochondria were permeabilized and blocked in TritonX-100 (AppliChem, Darmstadt, Germany) with 5% goat serum for 30 min. Ribosomal protein S6 (54D2) was stained with primary conjugated mouse mAb (S6 ribosomal protein (54D2, Alexa Fluor® 488 conjugate), #5317, Cell Signaling Technology Inc., Danvers, MA) in 1:10000 dilution.

The actin cytoskeleton was visualized by incubating 1:10 phalloidin rhodamine stock dilution (Sigma-Aldrich, St. Louis, MI, USA) for 10 min at RT.

As of last, nuclear counterstaining was performed for all samples with DAPI (1:1000 dilution of 1 mM stock, Sigma-Aldrich, St. Louis, MI, USA) for 15 min at RT. DAPI is widely used for DNA visualization. It binds strongly to adenosine-thymidine (A-T)

rich regions of nuclear DNA via electrostatic interactions (Hayashi et al., 1992, Tarnowski et al., 1991). DAPI staining followed by mounting with Mowiol 4-88 on 8 chamber ibidi slides. The mountant was allowed to cure for at least 48 h. Confocal laser scanning microscopy (LSM 780, Carl Zeiss, Jena, Germany) with ZEN software Version 2.3 was used for image acquisition. The data was processed by GraphPad Prism Software (GraphPad Software).

2. RESULTS AND DISCUSSION

2.1 Cellular growth and cell size analysis

CHO DP-12 cells exposed to osmolality elevation show proliferation depression and cell size increase

After an initial adaptation phase, the CHO DP-12 cells start to grow exponentially (Figure **1**, a). 72 h post-seeding, 6 ml of supplemented feed was added (pure feed: 830 mOsmol/kg) to the "feed" condition (in the following, referred to as "F") or growth medium (306 mOsmol/kg) to the control condition (in the following referred to as "C"). The osmolality in the control condition remained unchanged throughout the cultivation, except for a typical decrease to about 250 mOsm/kg on day eight due to the depletion of substrates in the medium. The addition of the supplemented feed caused a step-wise increase of osmolality in F from the initial physiological level of 304±2 mOsm/kg to 545±3 mOsm/kg in four increments (Figure ***1***, b). The first two feedings bringing the mean osmolality up to 460±3 mOsm/kg were sufficient to induce complete proliferation arrest accompanied by the onset of cell size increase measured by Cedex AS 20. The distribution of the cell size populations on days two, four, six, and eight are shown in Figure ***1***, c. The cells in the feed condition reached an average diameter of 20.8±0.3 µm (the mean between days seven, and ten), which corresponds to the cell

volume of 3784.9±0.3 fL assuming a perfectly spherical form. Compared to the cells in control conditions (mean diameter days 7-10: 14.7±0.9 µm, volume 1329.2±1.2 fL) this corresponds to a 39% increase in cell diameter and x2.7 increase in cell volume. The data shown here were observed for at least three independent experiments following the same set-up. We found out that the cell size increase is a reversible phenomenon: CHO cells previously exposed to high osmolality feed return to their normal size of 15±2.0 µm and start proliferating normally upon return into physiological conditions within roughly 48 h (data not shown).

While mammalian cells significantly vary in size between the cell types, the cell size is narrowly constrained for the given cell type and cultivation condition (Cadart et al., 2018). During the logarithmic phase in a normal fed-batch, the cell size is narrowly distributed between 12.5 and 18.0 µm (Figure ***1***, c, blue bars), obviously showing a consistent pattern between cell cycle duration, biomass accumulation, and cell doublings. These processes are synergistically regulated and depend on each other (Cadart et al., 2018). On day eight, almost 80% of the cells are confined between 12.5 and 18.0 µm, 11% between 18.0 and 25.0 µm, and none exhibiting a diameter over 25.0 µm. Cells in the "feed" on the contrary, show a broader cell-size distribution pattern (Figure ***1***, c, red bars), where some cells get very large (over 25.0 µm), the main population shifts progressively to the 18.0-25.0 µm gate resulting in the shift of the average diameter. Some cells still retain their original typical cell size of 12.5 – 18.0 µm. A similar broadening of cell-size distribution patterns for CHO cells exposed to osmolality increase has been reported previously (Kiehl et al., 2011, Pan et al., 2017). However, even if the osmolality gradient was set much steeper, achieving about 630±7 mOsm/kg after four feedings, the average CHO DP-12 cell diameter did not exceed the maximum of 22.3±0.3 µm (unpublished data).

Cell cycle distribution analysis shows no increase in DNA content in "feed" cells

Further, we studied the distribution of DNA content in non-proliferating (hyperosmolality exposed) and normally growing CHO cells. To do so, we used a classical cell cycle distribution experiment, based on the measurement of propidium iodide-stained DNA fluorescence via flow cytometry. We were able to observe a clear difference on days four and five between rapidly proliferating cells in "control" and VCD-stable cells in "feed" (see Figure 1, e): the cells under control condition show a smooth transition from haploid (1N) G_0/G_1 to diploid (2N) G_2/M phase with clearly distinguishable S-phase. The cells in the feed seem to be arrested either in G_0/G_1 or in the G_2/M phase but lack the transition S-phase. The accumulation of 1N and 2N cells combined with concomitant depletion of the S-phase has been shown for yeast (Alexander et al., 2001) and recently for the CHO cells (Pan et al., 2019), exposed to osmotic pressure. In both cases, the cells increased in diameter and did not proliferate. There seems to be no trend of accumulating >2N DNA cells specific to one of the conditions. On day seven, the number of polyploid cells or unresolved cell doublets (aggregates) does not vary significantly between "control" and "feed" populations. Thus, we conclude that the cells in "feed" increase in diameter, but do not generate more DNA.

Interpretation of the cell size distribution based on flow cytometry measurement of propidium-iodide stained cells can be only done with caution. Before the DNA staining, the cells were fixed with 70% EtOH and permeabilized with 0.1% saponin. Although fixation with ethanol gives the best correlation of macroscopic features to the live cells (Li et al., 2017), permeabilization with saponin might alter their properties as measured in forward scatter (FSC, correlates with cell size) and side scatter (SSC, cells´ granularity).

Summarizing the above, upon exposure to high-osmolality feed CHO DP-12 cells endure proliferation arrest coinciding with gradually progressing cell size increase. The cells in "feed" show an x2.7 volume increase, but no increase in DNA content measured by propidium DNA staining via flow cytometry. The cells in "feed" reside either in G_0/G_1 or in the G_2/M phase but lack the transition S-phase. Several studies including CHO (Bi et al., 2004), human cells (Demidenko & Blagosklonny, 2008), and budding yeast (Neurohr et al., 2019) show that the cells continue to increase in size when cell cycle progression is blocked by either external or internal factors. We suppose that cell exposure to osmolality well above the physiological level promotes cell cycle and DNA replication arrest. This, in turn, causes an increase in cell size due to continued biomass production. Further mechanisms of cells' adaptation to osmolality increase include modulation of mitochondrial activity and altering membrane characteristics, discussed in the next chapters.

2.2 Assessment of mitochondrial activity and apoptosis induction via flow cytometry

High osmolality feed causes an increase in mitochondrial membrane potential

Mitochondria are the main oxygen consumer of the cell, using 85% of the total amount assimilated by the cell for electron transport (Ademowo et al., 2017, Chan, 2006).

Mitochondria-specific marker MitoTracker chloromethyl-X-rosamine (CMXRos) used in this study is a thiol-reactive lipophilic dye, able to passively diffuse across the plasma membrane and to accumulate in active mitochondria (Poot et al., 1996). Its ability to do so depends on mitochondrial membrane potential $\Delta\Psi m$: A more negative or polarised $\Delta\Psi m$ will accumulate more dye, and vice versa. The membrane potential is in turn an important mitochondrial physiological parameter and relates to the cells´ ability to

produce ATP, which is decisive for oxygen consumption in mitochondria. CMXRos is a nontoxic sensitive indicator of relative changes in ΔΨm (Pendergrass et al., 2004). It also has the advantage to be retained in the organelles after methanol fixation (Macho et al., 1996).

To sum up the above, the fluorescence intensity measured at the emission maximum of the MitoTracker CMXRos dye (561 nm) shows a combined effect of 1) mitochondrial membrane potential ΔΨm which indicates health and activity of mitochondria in the cell (Cottet-Rousselle et al., 2011), and 2) is entangled with the amount of the mitochondrial membrane (Gilson et al., 2003), which depends on size and number of the mitochondria in the cell.

As an elevation of cell-specific oxygen uptake in CHO cells exposed to osmotic stress has been reported previously (Pan et al., 2017), we hypothesized that mitochondrial functionality plays a prominent role in cells´ adaptation and survival in our set-up. Thus, we anticipated finding a difference in mitochondrial fluorescence based on the MitoTracker-labeling between the “feed” and “control” conditions.

In agreement with our hypothesis, we detected a significant ($p \leq 0.05$) fluorescence increase of MitoTracker-labeled mitochondria using flow-cytometry on days six and eight in the “feed” compared with the same time points in the “control” condition (Figure ***2*** panel a). We used an unpaired parametric two-tailed *t*-test for statistical evaluation. The *t*-test analyzes the differences between two groups based on a single, ordinal variable, assuming a normal distribution in the population (McKnight & Najab, 2010), which seems to be appropriate based on the measured distribution of fluorescence intensity (Figure ***2***, panel a, 1-3). Almost a half (46.6±15.6% on day six and 49.7%±6.4% on day eight) of the cells was gated “bright” in the “feed” compared

to almost non (2.1±1.9% for day six and 1.9±0.4% for day eight) in the control (Figure ***2***, panel a, 3). The "dim" gate showed the complementary inverted distribution (Figure ***2***, panel a, 3). Thus, the observed cell volume gain in the "feed" is coupled with pronounced mitochondrial mass and activity boost.

The increase of mitochondrial activity observed in the "feed" on day six and eight of the fed-batch process is accompanied by its concomitant reduction in the "control" condition. As mitochondrial activity is correlated with protein synthesis and cell growth (Wahrheit et al., 2014), the observed decrease of mitochondrial fluorescence in "control" is most likely a manifestation of lower ATP synthesis rate by the cells entering a stationary phase. Such development of mitochondrial activity has been previously observed for hybridoma (Al-Rubeai et al., 1991) and CHO cells (Zagari et al., 2013) in the batch mode.

An existing study (Bi et al., 2004) reporting a similar cell volume increase of CHO cells as observed in our experiment attributed the increment of mitochondrial fluorescence measured via MitoTracker FM Green fluorescence (contrary to the CMXRos, this dye is membrane-potential independent and correlates solely to mitochondrial mass) to the biogenesis of the new organelles and not to the activation of the existing ones. The authors also detected an increased metabolic activity of the mitochondria measured via dehydrogenase activity assay.

We suggest that our observation of increased mitochondrial fluorescence is most likely rooted in increased $\Delta\Psi m$ polarization and/or size growth of existing mitochondria combined with the biogenesis of the new organelles. Although we recognize the limitation of the flow cytometry analysis to pinpoint the exact contributions of each of

these effects, we believe that the up-regulation of mitochondria plays a central role in CHO cellular response to osmotic stress.

High-osmolality feed does not induce apoptosis in CHO DP-12 cells

To assess if the high-osmolality feed induces apoptosis in the CHO cells, we performed a caspase 3/7 activation assay (Figure 2, panel b). The activation of effector caspases 3 and 7 is a rapid event marking the onset of the apoptotic death (Tyas et al., 2000). It can be detected by measuring a fluorometric substrate cleavage (fluorophore-conjugated DEVD-peptide) by activated caspases 3 and 7 using flow cytometry.

The available research data addressing apoptosis induction in osmotically stressed cells is rather ambivalent. Whether the cells die via apoptosis or necrosis upon exposure to hyper- or hypoosmotic pressure seems to depend on the cell type, severity of stress (Kim & Lee, 2002, Tao et al., 2002), and the agent used. Increased cellular apoptosis was observed in hybridoma (deZengotita et al., 2002), canine epithelial cells (Terada et al., 2001), and in antibody and EPO-producing rCHO cells (Han et al., 2010, Wang et al., 2012) where osmolality increase was induced by the addition of NaCl solution. On the other hand, kidney cells exhibited no apoptosis induction (Mak & Kültz, 2004) at 540 mOsm/kg, but did so upon even further (650-700 mOsm/kg) osmolality increase.

The cells stained with 5 μM CellEvent® Caspase-3/7 Green Detection Reagent were not washed throughout the whole preparation process to preserve a native cell distribution. The flow cytometry analysis was conducted immediately after the incubation. The gate was set to exclude DAPI-positive dead cells, cell fragments, and unresolved cell doublets.

To exclude false-negative results, we verified the proper functionality of the assay in CHO DP-12 cells by inducing apoptosis with staurosporine (0.5 μM, 5 h incubation)

and applying the same staining and measurement protocol. We were able to detect about 30% of bright green cells (data not shown) confirming that observing no caspase 3/7 activation is not caused by invalid assay performance.

Our experimental results show indeed no signs of early apoptosis during the fed-batch cultivation of CHO DP-12 cells throughout all three sampling points (days two, six, and eight) in both control and feed conditions (n = 3 for each condition). No shift in fluorescence was observed in the FL-1 channel at 514 nm between stained and unstained cell populations (see Figure ***2***, b), affirming no programmed cell death induction either in osmotically stressed "feed" or normally cultivated "control" cells. This result was reproduced throughout at least three independent cultivations.

Apoptosis is a very multifarious and complex process. Its regulation, signaling, and anti-apoptotic engineering have been extensively studied in CHO cells and are regularly reviewed (Henry et al., 2020, Krampe & Al-Rubeai, 2010) in literature. Briefly consolidating the available data, it seems that apoptosis is not an imminent part of osmolality-induced stress response in general but is rather activated if e.g. vital nutrients are concurrently depleted in the culture (Han et al., 2011, Hwang & Lee, 2008). As we used a complete supplemented feed solution specifically designed for CHO cells, we presume that no substrate limitations occurred in the feed condition during the fed-batch process, thus explaining why the CHO DP-12 cells did not induce apoptosis in our experiment.

2.3 Single-cell observation via confocal microscopy and ICC

We next validated our flow cytometry analysis by analyzing the mitochondria amount and activity in CHO DP-12 cells exposed to high osmolality feed conditions using ICC. Here, the CHO DP-12 cells cultivated in a fed-batch mode with high osmolality feed

added on days three, four, five, and six were sampled on days two, six, and eight, stained with 200 nM MitoTracker® Red CMXRos (Cell Signalling Technology Inc., Danvers, MA, USA) and imaged using confocal laser scanning microscopy (Figure 3 a and b). The first sample (day two) served as a reference point prior to feed or supplemented medium addition. As expected, no differences in the amount of mitochondrial fluorescence in CHO cells were observed (two days of culture, Figure ***3***, a). On day eight (Figure ***3***, b), only a slight tendency towards mitochondrial fluorescence increase can be observed in "feed" cells with respect to "control" (Figure ***3***, b). One should keep in mind that cells lose their mitochondrial membrane potential upon fixation and permeabilization, thus the dye can be used primarily for mitochondrial localization and rough abundance determination. Besides, we have performed antibody-based staining of S6 ribosomal protein (54D2) to detect its total endogenous level in cells. Ribosomal protein S6 is a part of the ribosomal S40 subunit. Its phosphorylation has been reported to be related to cell size regulation and cell cycle progression (Ruvinsky et al., 2005; Ruvinsky and Meyuhas, 2006). Although we did measure a slight S6 abundance increase via LFQ tandem mass spectrometry (please refer to electronic supplementary material of the companion article: Hyperosmolality in CHO Culture: Effects on Proteome) ($\log_2$ fold change F vs. C D6 +0.11, D8 +0.01), this change is scarcely detectable by eye indicating that feed-based increase in cell size might be based on an S6-independent mechanism. The population-level cell size increase measured via Cedex AS20 was not observable via ICC because mounting the cells on the slide surface inhomogeneously altered their initial spherical form.

CHO DP-12 cells treated with high osmolality feed reveal significantly increased amounts of nuclei per cell

To evaluate the impact of high osmolality feed on cell nucleus morphology, CHO cells treated with feed for four and six days (days six and eight of the fed-batch) were stained with DAPI and phalloidin rhodamine. On reference day two (48 h after seeding) no significant changes in the number or shape of nuclei of CHO cells were observable (Figure ***4*** a, c). The cells displayed mostly uniform round-shaped single nuclei both in control and feed populations. On days six and eight the CHO cells in the feed condition showed a highly significant increase in the number of nuclei or the presence of micronuclei (Figure ***4*** b, arrows) compared to the control condition (Figure ***4*** b, arrowheads).

Micronuclei are formed during cell division when acentric chromosome fragments or whole chromosomes that were not incorporated into the main nuclei during cell division are enclosed by a nuclear membrane (Fenech & Morley, 1985). Micronuclei were reported to be a biomarker for Multipolar anaphase, genotoxic effects, and chromosome aberration (Norppa, 2003, Pastor et al., 2009). In accordance with our findings, changes in osmolality were already shown to cause the formation of micronuclei (Fenech et al., 2010, Henderson et al., 2000, Meintières & Marzin, 2004). In particular, Meintières and Marzin observed the exposure of CTLL-2 cells to high osmolarity by NaCl to result in the formation of micronuclei. To the best of our knowledge, the formation of micronuclei in CHO cells exposed to osmolality increase has not been observed previously. Meintières and Marzin further demonstrated micronuclei formation to be accompanied by increased apoptosis of CTLL-2 cells exposed to NaCl (Meintières & Marzin, 2004). On the contrary, our present findings revealed an increased amount of micronuclei in CHO cells after high osmolality feed without induction of apoptosis (Figure ***2***, b).

We suppose that nuclear shape alterations in CHO cells are probably a direct result of osmotically induced stress. A recent finding reveals that exposure to elevated ROS concentrations induces nuclear shape alterations including fragmentation and folding due to aberrations in the reassembly of the nuclear envelope in cancer cells (Ahn et al., 2019). The authors discovered that the cells are most vulnerable during mitosis, so it is unclear if the structures we observed on days six and eight in the "feed" condition are artifacts of uncompleted mitotic division disrupted by osmolality increase on day three/four or if these have evolved during the fed-batch gradually with time.

3. CONCLUSIONS

The increased osmolality response of mammalian cells differs principally from that of bacteria. It was shown that *E.coli* cells lose water and decrease in the cell size proportional to extrinsic osmolality increase (Dai & Zhu, 2018). The mammalian cells on the contrary show the expected cell volume decrease due to cytoplasmic fluids loss only in the first hours of osmotic shock. After this, the cells seize to proliferate but continue to produce biomass, achieving significantly larger cell diameter and cell volume.

Although effects of osmolality change in respect to productivity and growth of CHO cells have been a topic of several studies in the past years (Kiehl et al., 2011, Pan et al., 2017, Shen et al., 2010, Takagi et al., 2000), we used a combined multi-level approach for the first time. We have conducted a flow cytometry analysis to access mitochondrial activity and apoptosis induction. Also, the cells were visualized with different stains via confocal microscopy to get an insight into the phenotype of the cells subjected to osmotic stress. Our results suggest that osmolality increase causes substantial adaptation effects, involving mitochondria activation, proliferation arrest, cell-size

increase, and formation of multiple nuclei in some cells. We also showed that hyperosmolality does not necessarily trigger an increase in cell-specific productivity (qP). Further, we conducted an LFQ proteome analysis to highlight the significantly regulated protein clusters via statistic evaluation based on their molecular function. The results of this analysis are discussed in a companion article “Hyperosmolality in CHO Culture: Effects on Proteome”.

ACKNOWLEDGMENTS

The authors would like to thank Nele v. Vegesack for her assistance with preliminary experiments and live stains sample preparation. This work was funded by Bielefeld University. Nadiya Romanova is supported by a Grant for women in the orientation phase initiated by the Equal Opportunities Commission of the Faculty of Technology, Bielefeld University.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

N. Romanova designed the experiments, carried out cell cultivation, sample preparation, analyzed and interpreted the data, and wrote the manuscript. T. Niemann contributed to experiment design, performed the flow cytometry measurements (live stains), immunocytochemical sample preparation and analysis, evaluated the data, and wrote the manuscript. J. FW Greiner contributed to data analysis and interpretation, flow cytometry troubleshooting, and preparation of the manuscript. B. Kaltschmidt and C. Kaltschmidt contributed to experiment design and supervised the project. T. Noll conceived an initial project idea, acquired funding and supervised the project. All authors read and edited the manuscript.

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Tables

Table 1. Selection of staining reagents and their targets in CHO cells used to characterize cell size increase on single-cell plane

Target	Staining reagent	Used Concentration	Excitation wavelength
Mitochondria	MitoTracker® Red CMXRos†	200 nM	561 nm
Apoptosis activation	CellEvent® Caspase-3/7 Green Detection Reagent‡	5 μM	488/514 nm
Ribosomes	S6 Ribosomal Protein (54D2) Mouse mAb Alexa Fluor ® 488 Conjugate antibody†	1:400 dilution	488 nm

† Cell Signalling Technology, Cambridge, UK

‡ Thermo Fisher Scientific, Waltham. MA

5. FIGURE LEGENDS

Figure 1 Fed-batch cultivation of CHO DP-12 cells, based on three biological replicates per condition. (control vs. feed) **a)** Viable cell density VCD, [$\times 10^5$ cells/ml], and viability [%] for control and feed conditions. Black vertical lines indicate the addition of 6 ml supplemented feed to "feed" shakers and growth medium to "control" shakers; **b)** Osmolality [mOsm/kg] and mean cell diameter [µm] during the fed-batch cultivation; **c)** The cell size distribution measured by Cedex AS 20 during the fed-batch cultivation of the DP-12 cells, attributed to three gates 12.5-18 µm, 18-25 µm, >25 µm. **d)** Average antibody concentration [mg/ml] and cell-specific productivity qP [pg/cell×day] during the fed-batch cultivation of CHO DP-12 cells, based on three biological replicates per condition. The antibody concentrations after 24 and 48 h are below the limit of detection and thus could not be estimated.; **e)** The cell cycle distribution analysis on days two, four, five, and seven for the fed-batch cultivation of CHO DP-12 cells. The upper row shows the control condition and the lower row the feed condition. For each condition and time point, the propidium iodide (PI) stain intensity (FL2-H) is shown as a function of a linear count histogram. FL-2H indicates the amount of DNA in the cell. The events measured at intensity over 2 000 are most likely to be caused by cell aggregates.

Figure 2 a) The flow cytometry analysis of mitochondria activity on days two, six, and eight for the fed-batch cultivation of CHO DP-12 cells. 1) Difference in mitochondrial fluorescence measured by flow cytometry on days two, six, and eight. The X-axis - log-scale FL6-A fluorescence intensity (corresponding to mitochondrial fluorescence); the Y-axis - the linear events count. Unstained (grey) and MitoTracker®CMXRos-stained (blue for control and red for feed) cell populations for one biological replicate are shown as an example for each time point. Cell debris (based on FSC-A vs. SSC-A dot plot), cell aggregates (based on FSC-H vs. FSC-A diagonal plot), and dead cells (based on DAPI nuclear stain, measured in FL1-A channel) were gated out. Gates "dim" and "bright" were applied uniformly to all measured samples. 2), 3) Graphical representation of mitochondrial fluorescence data showing a percentage of the cells in the "bright" and "dim" gate of FL6-A ($n = 3$). Statistical evaluation was performed by an unpaired parametric two-tailed *t*-test (*p-value < 0.05; ***p-value < 0.0005); **b)** The flow cytometry analysis of caspase 3/7 activation on days two, six and eight for the fed-batch cultivation of CHO DP-12 cells. The graphs are plotted in log-scale FL2-A (Caspase fluorescence, X-axis) against the linear events count (Y-axis). Unstained (grey) and Caspase 3/7 Green detection Reagent-stained (blue for control and red for feed) cell populations for one biological replicate is shown as an example for each time point. Cell debris was gated out based on FSC-A vs. SSC-A dot plot, cell aggregates based on FSC-H vs. FSC-A diagonal plot and dead cells based on DAPI nuclear stain, measured in FL1-A channel.

Figure 3 Immunocytochemical analysis of mitochondrial and ribosomal staining on days two and eight under fed-batch conditions of CHO-DP12 cells exposed to high osmolality ("feed") or without osmotic changes ("control"). a), b) Cells on days two and eight of the fed-batch cultivation centrifuged down on µ-slide 8 well chamber slides and stained with MitoTracker® Red CMXRos, the primary antibody against 40S ribosomal protein S6 and DAPI. The primary antibody was visualized with secondary Antibody Alexa Fluor 488.

Figure 4 Immunocytochemical analysis of cytoskeleton and nuclear morphology across three cultivation time points for fed-batch cultivation of CHO-DP12 cells to high osmolality feed ("feed") or without osmotic change ("control"). a), b) Two and eight days of cultivation centrifuged down on µ-slide 8 well chamber slides and stained with phalloidin rhodamine and DAPI. c) Quantification and comparison of micronuclei formation on day two, six, and eight between feed and control conditions; statistical significance by an unpaired parametric two-tailed *t*-test with the statistically significant threshold of *p-value < 0.05; ***p-value < 0.0005.