RESEARCH NOTE

Detrimental effect of zwitterionic buffers on lysosomal homeostasis in cell lines and iPSC-derived neurons [version 1; peer review: 3 approved]

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Abstract
Good’s buffers are commonly used for cell culture and, although developed to have minimal to no biological impact, they cause alterations in cellular processes such as autophagy and lysosomal enzyme activity. Using Chinese hamster ovary cells and induced pluripotent stem cell-derived neurons, this study explores the effect of zwitterionic buffers, specifically HEPES, on lysosomal volume and Ca²⁺ levels. Certain zwitterionic buffers lead to lysosomal expansion and reduced lysosomal Ca²⁺. Care should be taken when selecting buffers for growth media to avoid detrimental impacts on lysosomal function.

Keywords
Ca²⁺, HEPES, iPSC, lysosomal disease, lysosome, neuron, zwitterionic buffer

This article is included in the Alzheimer's Research UK gateway.

Open Peer Review

Reviewer Status

Invited Reviewers

1. Stephane Lefrancois, National Institute of Scientific Research, Laval, Canada
2. Johannes Aerts, Leiden Institute of Chemistry, University of Leiden, Leiden, The Netherlands
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4. Sara Mole, University College London, London, UK

Any reports and responses or comments on the article can be found at the end of the article.
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Author roles: Cook SR: Data Curation, Formal Analysis, Investigation, Methodology, Writing – Review & Editing; Badell-Grau RA: Data Curation, Formal Analysis, Methodology, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; Kirkham ED: Data Curation, Formal Analysis, Investigation, Methodology, Visualization, Writing – Review & Editing; Jones KM: Investigation, Methodology, Resources, Writing – Review & Editing; Kelly BP: Investigation, Resources, Writing – Review & Editing; Winston J: Investigation, Resources; Waller-Evans H: Funding Acquisition, Project Administration, Supervision, Writing – Review & Editing; Allen ND: Methodology, Project Administration, Resources, Supervision, Writing – Review & Editing; Lloyd-Evans E: Conceptualization, Data Curation, Funding Acquisition, Investigation, Methodology, Project Administration, Supervision, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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NPCs were expanded in ADF with 2% NeuroBrew 21 with SB431542 (10 µM, Abcam) and IWR1 (1.5 µM, Tocris). retinoic acid (Miltenyi), LDN193189 (1 µM, Stemgent), streptomycin (Life Technologies), 2% NeuroBrew 21 without in Advanced DMEM/F-12 (ADF) with GlutaMAX, penicillin/streptomycin, and alteration to autophagy and lysosomal enzyme activity in cultured cells. 

Lysosomes are acidic organelles, known as the recycling centre of the cell, since they breakdown cellular material. They also have important roles in cellular processes, including plasma membrane repair and cellular signalling as the second largest intracellular Ca²⁺ store. Lysosomal dysfunction is a component of multiple diseases including Alzheimer’s, Parkinson’s and ~70 inherited lysosomal storage diseases. Considering the reported impact of HEPES on lysosomal enzymes, it is important to understand its effects, as well as other zwitterionic buffers, on lysosomal functions.

This study describes the effect of HEPES on lysosomal morphology and Ca²⁺ levels in control cells and those null for the lysosomal protein NPC1, whose function is lost in the lysosomal storage disease Niemann-Pick Type C (NPC). The findings highlight the importance of understanding the impact of growth media components on lysosomal functions.

**Methods**

**Cells**

Chinese hamster ovary (CHO) control H1 and NPC1-null M12 cells were grown as monolayers at 37°C/5% CO₂, in Dulbecco’s Modified Eagle’s Medium (DMEM)/F-12 (Thermofisher) with 1% L-glutamine (Lonza), 10% heat-inactivated foetal bovine serum (Sigma/Pan Biotech) either with or without HEPES/other zwitterionic buffer at pH 7.4 (Thermofisher/Lonza).

Control induced pluripotent stem cell (iPSC)-derived neural progenitor cells (NPCs) were cultured on vitronectin-coated 6-well plates with E8 flex medium (Life Technologies) at 37°C/5% CO₂. Neural induction proceeded according to previous methods with modifications. Briefly, NPCs were derived in Advanced DMEM/F-12 (ADF) with GlutaMAX, penicillin/streptomycin (Life Technologies), 2% NeuroBrew 21 without retinoic acid (Milenyi), LDN193189 (1 µM, Stemgent), SB431542 (10 µM, Abcam) and IWR1 (1.5 µM, Tocris). NPCs were expanded in ADF with 2% NeuroBrew 21 with retinoic acid (Milenyi Biotec) and 10 ng/mL basic fibroblast growth factor. NPCs were terminally differentiated in SynaptoJuiceA (HEPES-free) for 7-days, followed by two weeks in SynaptoJuiceB (5.5 mM HEPES) according to. Neurons were maintained in SynaptoJuiceB, both with and without additional 10 mM HEPES for 7 days.

**Buffers**

All buffers (MOPS, PIPES, MES, PPB) were purchased from Sigma-Aldrich apart from MES (Thermofisher/Lonza) and Tris (Roche). With the exception of HEPES, which was purchased as a pre-made 1 M solution (pH 7.4), all buffers were made as 1 M stock solutions in mgH₂O (or 1 M NaOH in mgH₂O for PIPES), adjusted to pH 7.4 and filtered sterilised through a 0.22 µm filter. PPB was adjusted to pH 7.4 by combining 1 M solutions of monobasic dihydrogen phosphate and dibasic monohydrogen phosphate. Buffers were added to cell culture media to a final concentration of 10 mM unless otherwise stated.

**Lysosomal measurements**

Lysosomes were visualised in live cells in chamber slides (Ibidi) using 300 nM LysoTracker red or green (Life Technologies) in Dulbecco’s modified phosphate buffered saline (DPBS) for 15-minutes at room temperature, washed three times with DPBS, and imaged using a Zeiss Axio Observer inverted microscope with Colibri LED light source and Zeiss Mrm CCD camera with Axiovision 4.8 software. Lysosomal area per cell was measured from LysoTracker fluorescence images in ImageJ (Figure 1A & B). Lysosomes were imaged in Corning CellBIND 96-well plates (0.8x10⁵ cells/well) using a SpectraMax® Gemini microplate reader (Molecular Devices). Lysosomal measurements were done as described but with minor modifications for neurons, which were loaded with 1 µM Fura-2, AM (Stratech) without Pluronic F-127. Cells were imaged in Hank’s balanced salt solution (HBBS; 1 mM HEPES pH7.4, 10 µM CaCl₂ and 1 mM MgCl₂) using a Zeiss Axiovert 35 microscope with Cairn Optospin filter exchanger, Orca Flash 4.0 sCMOS camera and MetaFluor 7.10 software. For all experiments, ionomycin (Merck, 2 µM) was added to clamp intracellular Ca²⁺ stores followed by 500 µM Gly-Phe-β-naphthylamide (GPN, Abcam) to release lysosomal 

**Statistical analysis**

All statistical analyses were performed in GraphPad Prism 8 software with data analysed by two-way ANOVA with Tukey’s post-hoc test or unpaired t-test as appropriate and where indicated in the figure legends.

**Results**

In agreement with previous findings of lysosomal enzyme dysfunction, we observed HEPES-mediated lysosomal dysfunction in control CHO-H1 cells that was exacerbated at high cell confluency. Namely, a concentration-dependent expansion of the lysosomal system following 3-days growth in HEPES-containing buffer observed using LysoTracker (Figure 1A & B). Having confirmed this effect, we determined whether other zwitterionic buffers triggered similar effects. At a
Figure 1. Changes in lysosomal area in cells grown in zwitterionic buffered media. (A) Representative images of control CHO-H1 cells loaded with LysoTracker green following 3-day treatment with the indicated concentrations of HEPES buffer. (B) Quantitative analysis of LysoTracker fluorescence from (A) as lysosomal area per cell, N=3–4 (9 cells analysed per repeat). (C) Representative images of CHO-H1 cells loaded with LysoTracker green following treatment for 3-days with 10 mM of the indicated buffers. PPB is potassium phosphate buffer. (D) Quantitative analysis of LysoTracker fluorescence from (C) as lysosomal area per cell, N=3–4 (8–9 cells analysed per repeat). (E) Fluorescence plate assay of control CHO-H1 cells and NPC1-null CHO-M12 cells loaded with LysoTracker green following 12-month growth in HEPES buffered medium, N=8. (A) and (C) Scale bars = 10 µm. (*p<0.05, **p<0.001, ***p<0.0001, two-way Anova tests, post hoc Tukey's).
buffer concentration commonly found in growth media (10 mM), only PIPES, out of the six buffers tested, increased lysosomal area in control CHO-H1 cells over the 3-day treatment, that was also exacerbated by high cell confluency (Figure 1C & D).

To determine the long-term effects of growth in HEPES-containing media (10 mM), control CHO-H1 and the NPC1-null CHO-M12 cells were grown in this media for 12-months. When grown in HEPES-free media, there is a 4.8-fold increase in LysoTracker florescence, measured using a plate reader, in the lysosomal disease CHO-M12 cells, compared to control CHO-H1. Following 12 months of growth in media with HEPES, we observed no further increase in LysoTracker staining in NPC1-null CHO-M12 cells, whereas we observed a 2.8-fold increase in LysoTracker fluorescence in control CHO-H1 compared with control cells grown in HEPES-free media (Figure 1E). This illustrates that growth in HEPES-supplemented media impacts upon healthy lysosomal function and reduces the difference between control and lysosomal disease cells. This observation may have particular importance for cells requiring long-term growth in buffered media (e.g., iPSC-neurons).

Therefore, we tested the effect of HEPES supplementation of SynaptoJuiceB on iPSC-neurons in culture for 7 days. Again, we observed an expansion of the lysosomal system (Figure 2A). Because zwitterionic buffers may act as a “proton sponge”, affecting both the volume and ion balance of lysosomes13, particularly lysosomal Ca²⁺ content which is dependent on lysosomal acidification14, we measured lysosomal Ca²⁺ content in these neurons. We observed significantly reduced lysosomal Ca²⁺ (2.2-fold) in neurons grown in the presence of HEPES.

![Figure 2](image_url)

**Figure 2.** Growth of iPSC-derived neurons in HEPES containing media results in altered lysosomal Ca²⁺ and causes lysosomal expansion. (A) Representative images of iPSC-derived neurons treated for 7 days in media containing 10 mM HEPES. Phase contrast microscopy images show location of neuronal cell bodies. Scale bar = 10 μm, N=3. (B) Following 7-day treatment in HEPES, lysosomal Ca²⁺ release, triggered by addition of 500 μM GPN, to induce osmotic lysis, after ionomycin to clamp other intracellular Ca²⁺ stores, was measured in iPSC-derived neurons, N=4 (7–14 cells analysed per repeat). (Ci) and (Cii) are Representative traces of Ca²⁺ release quantified in (B). (*p<0.05, unpaired t-test).
of 10 mM HEPES for 7 days compared to those grown without HEPES (Figure 2B). Raw data underlying this study are available at Figshare13.

Discussion and conclusions
Our findings indicate that lysosomal expansion occurs after both short- and long-term culture in HEPES-buffered media and is exacerbated at higher cellular confluency. Moreover, this expansion impacts lysosomal function, namely lysosomal ion signalling in the form of reduced lysosomal Ca\(^{2+}\) content and is consistent with previous report of altered lysosomal glucosylceramidase activity in cells grown in HEPES1. Together, these data suggest that HEPES operates as a lysosomal proton sponge15,16. These observations provide a significant note of caution for lysosomal researchers, potentially impacting on lysosomal biochemical experiments such as measurement of pH17 or lysosomal purification methods18. Not all zwitterionic buffers have the same effects, only PIPES was also detrimental to lysosomal function, suggesting other zwitterionic buffers may be appropriate HEPES substitutes. Regardless, stringent consideration must be spent on buffer selection for relevant lysosomal studies.

Data availability
Underlying data
Figshare: Detrimental effect of zwitterionic buffers on lysosomal homeostasis in cell lines and iPSC-derived neurons. https://doi.org/10.6084/m9.figshare.12218441.v113.

This project contains the following underlying data:

- **Figure 1b** HEPES concentration effect on lysosomal area (CSV). (Effect of different HEPES concentrations on lysosomal area.)

- **Figure 1d** Effect of zwitterionic buffers on lysosomal area (CSV). (Effect of each zwitterionic buffer on lysosomal area.)

- **Figure 1e** Effect of long term HEPES growth on LysoTracker fluorescence (CSV). (Fluorescence levels in CHO-H1 and NPC1-null CHO-M12 cells grown in HEPES for 12 months.)

- **HEPES Effect on iPSC neurons Fura 2 GPN Ca\(^{2+}\) peak height data fig2b (CSV).** (Effect of 7-day HEPES incubation on Ca\(^{2+}\) release in iPSC-derived neurons.)

- Untreated Iono GPN Fura 2 trace raw data fig2ci (CSV). (Raw Ca\(^{2+}\) release quantified from the above experiment, no HEPES.)

- 10 mM Heps Iono GPN Fura 2 trace raw data fig2cii. (Raw Ca\(^{2+}\) release quantified from the above experiment, 10 mM HEPES.)

- Raw microscopy images (28 images; TIF).

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Author contributions
SRC and EDK performed experiments and assisted with figure preparation. RABG wrote the manuscript, analysed the data, prepared the figures and performed statistical analyses. KMI, BPK, JW and NDA grew and provided the NPCs used. HWE provided supervision and assisted with manuscript preparation. ELE designed the study, performed some of the experiments, provided supervision, and co-wrote the manuscript.

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Version 1

Reviewer Report 11 June 2020

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Sara Mole
GOS Institute of Child Health and MRC Laboratory for Molecular Cell Biology, University College London, London, UK

This short report investigates the effect of commonly used buffers on the lysosome and of one buffer on calcium ion levels in normal CHO and NPC1 disease cells.

They observed an effect on lysosome area with increasing concentration of Hepes and examined an effect by other buffers at 10mM concentration. The effect was striking for Hepes and Pipes, and was more pronounced at higher cell confluency. Because some cell systems require longterm culturing, they also looked at cells that had been maintained in media for 12 months – they saw effects were further pronounced.

Looking at the effect of 7 days culturing of iPSC-neurons in 10 m Hepes they report an expansion of the lysosome although this is not quantitated. They also show GPN-induced calcium ion release form lysosomes is reduced, indicating the lysosome reservoir of calcium ions is affected.

This work is important as it shows that biological phenomena may be affected by media and length of time in this media, and that this needs to be taken onto account when designing and interpreting experiments.

Expansion of this work could examine other biological or organelle behaviour, and should take into account the concentrations o buffers used in common media.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Molecular Cell Biology, Lysosomal diseases

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 29 May 2020

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✅ **Johannes Aerts**
Leiden Institute of Chemistry, University of Leiden, Leiden, The Netherlands

**Marco van Eijk**
Department Medical Biochemistry, Leiden Institute of Chemistry, Leiden, The Netherlands

Together with a colleague the paper by Cook et al has been reviewed. The paper is clearly written and scientifically sound. We feel the paper nicely builds on the earlier work of Tol and colleagues (Autophagy 2018)\(^1\), who demonstrated HEPES triggered lysosomal biogenesis. The work presented here is relevant due to the tested cell types and it adds to the understanding how HEPES impacts lysosomes, now also revealing a Ca\(^{2+}\) response.

Some minor remarks, but the manuscript is suitable for passing peer review.

The minor questions open are:

- The commercial buffers normally are composed of 25mM HEPES. In the first figure 50 and 100mM have been used. Did authors verify toxicity?

- Have authors an idea if the MiT-TF family is involved in the studied cell types (small statement in discussion will do).
References
1. Tol MJ, van der Lienden MJC, Gabriel TL, Hagen JJ, et al.: HEPES activates a MiT/TFE-dependent lysosomal-autophagic gene network in cultured cells: A call for caution. *Autophagy*. 2018; 14 (3): 437-449 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Lysosomal storage disorders, obesity, metabolic inflammation.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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Stephane Lefrancois
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The manuscript by Cook at al aims to explore the effect of zwitterionic buffers on lysosome function and morphology.

This work is of importance to people in the field of lysosomal biology and other cell biologists.
performing experiments where the morphology and function of lysosomes are being considered. They confirmed the effects of HEPES of lysosomal function and they tested other buffers, finding an effect only with PIPES.

The results are well presented, properly quantified with statistical analysis. As the use of iPS cells is becoming more prominent in research, they extended their observation beyond CHO cells. They show the negative effects of HEPES buffer often used in cell culture media. These results should be considered by all biologists when culturing cells and how HEPES and other reagents could affect data.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
No source data required

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Cell biology, lysosome biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.