Chemical Research in Toxicology

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Communication

Cell via Cell Viability Assay Changes Cellular Metabolic Characteristics by Intervening with Glycolysis and Pentose Phosphate Pathway

Published as part of Chemical Research in Toxicology virtual special issue "Chemical Biology Approaches for Investigating Toxicological Mechanisms".

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Cite This: *Chem. Res. Toxicol.* 2024, 37, 208−211

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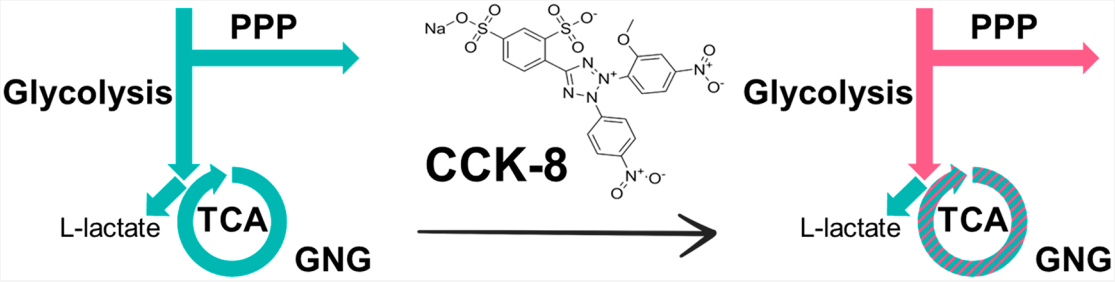

ABSTRACT: The Cell Counting Kit-8 (CCK-8) cell viability assay, also known as WST-8, is widely recognized for its nontoxic nature, making it suitable for further studies on treated cells. This practice is commonly observed in the field of tissue engineering. While live/dead imaging may not readily reveal macroscopic differences, our investigation has uncovered significant intracellular metabolic changes. Notably, we observed substantial down-regulation of metabolites within the glycolysis and pentose phosphate pathways. These metabolic alterations predominantly affect energy metabolism and may potentially impact the cellular redox environment. In light of these findings, we strongly recommend that researchers exercise caution when using cells treated with CCK-8 in subsequent experiments.

The Cell Counting Kit-8 (CCK-8) assay has been widely used in the assessment of cell viability and cytotoxicity. While CCK-8 is commonly employed as an end point assay, it is also frequently applied multiple times within the same experimental group, particularly in the fields of tissue engineering and material research (Figure S1).[1] Researchers frequently conduct multiple characterization experiments on the same sample in succession, often including the use of CCK-8, as a practical means of conserving both time and resources. The minimal toxicity exhibited by CCK-8 facilitates its application in treating cells for successive cell proliferation assays, including crystal violet assay, neutral red assay, or DNA fluorometric assay. However, it is important to acknowledge that uniform viability across different living cells might not precisely portray their cellular conditions. Moreover, the specific intracellular molecular changes induced by this assay in the treated cells remain uncertain.

The quantification of the cell metabolic activity is crucial for assessing cell viability. In the case of the CCK-8 assay, it relies on the detection of high NAD(P)H levels using tetrazolium salt. NAD(P)H levels directly correspond to dehydrogenase activity, thereby providing a measure of cellular metabolic activity.[2] As NAD(P)H is closely linked to metabolism, assays that rely on the depletion of NADPH are theorized to affect cellular metabolism, which, in turn, can affect cellular phenotype or function.

In this work, we employed a liquid chromatography−mass spectrometry (LC−MS)-based metabolomics workflow to identify metabolism pathways affected by CCK-8 treatments. In parallel to the CCK-8 assay, a live/dead staining assay and LDH assay were conducted to compare the status of the cells. The comparison of staining images between the CCK-8 treated group (named CCK) with the control group (named CNT) did not show a significant difference between them (Figure 1a and Figure S2). These results exemplify why researchers

Received: October 25, 2023
Revised: December 7, 2023
Accepted: December 14, 2023
Published: January 8, 2024

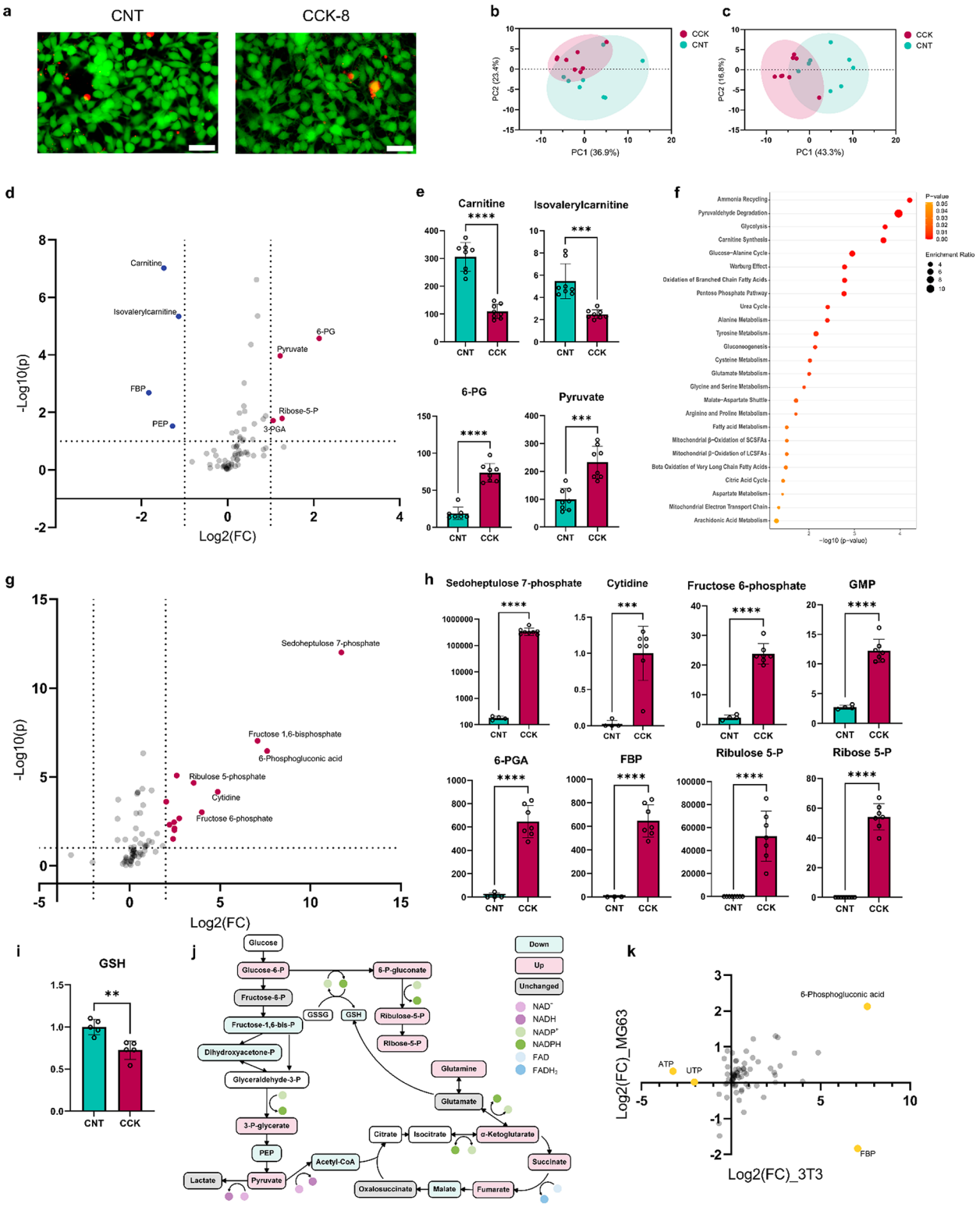

Figure 1. The changes of cells after treatment with Cell Counting Kit-8. (a) LIVE/DEAD staining of NIH/3T3 cells. Scale bar = 100 μm. (b, c) The PCA of MG-63 and NIH/3T3. (d) Volcano plots representing the significantly changed metabolites of MG-63 (Threshold FC > 2 or FC < 0.5, $p < 0.1$). (e) 4 metabolites that were significantly up-regulated and 4 that were down-regulated in MG-63 (unit pmol). (f) Pathway enrichment analysis was based on the metabolomic result of MG-63. The graph shows the top 25 pathways with significant alterations. (g) Volcano plots representing the significantly changed metabolites of 3T3. (h) The most significantly changed metabolites in NIH/3T3 cells. (i) GSH level of MG-63 cells treated with CCK-8 (unit pmol). (j) The metabolism map presented the mostly relevant pathways in energy production (MG-63, threshold Log2(FC) > 0.263034 was up-regulated, Log2(FC) < −0.263034 was down-regulated, 0.263034 > Log2(FC) > −0.263034 was considered as unchanged). (k) The comparison between the fold changes of MG-63 sarcoma cells and 3T3 fibroblasts (**$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

https://doi.org/10.1021/acs.chemrestox.3c00339
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typically deem cells treated with CCK-8 suitable for subsequent studies

Using LC−MS-based targeted metabolomics profiling, we obtained results for 74 metabolites from osteosarcoma (MG-63) cells and 78 metabolites from fibroblast (NIH/3T3) cells. MG-63 cells are commonly used as an osteoblastic or osteosarcoma models for tissue engineering studies, and NIH/3T3 cells are often used as a normal, noncancerous models in biomedical research.[3,4] Using these cells in our study provides information about the influence of CCK-8 on cancerous and normal cells.

These metabolites included amino acids, nucleotides, nucleosides, carboxylic acids, carnitines, and other organic acids (Figure S3). Principal component analysis (PCA) indicated the differences between CCK-8 treated cells and the controls (Figure 1b,c). In osteosarcoma cell lines, 8 metabolites were significantly changed (Figure 1d): 6 of them were relevant to glycolysis, and the other 2 were from the carnitine family (Figure 1d,e). In fibroblast cell lines, 14 metabolites were upregulated, of which mostly belong to glycolysis and pentose phosphate pathway (PPP) (Figure 1d).

The enrichment analysis (Figure 1e) highlighted that CCK-8 significantly impacted energy metabolism, particularly glycolysis, modifying cellular energy production. It also disrupted cellular homeostasis, influencing ammonia recycling and carnitine synthesis. Notably, the altered metabolites in the 3T3 cells were primarily linked to downstream glucose metabolism (Figure 1g).

The production of NAD(P)H is directly related to glucose metabolism pathways. This suggests that the glycolysis-relevant metabolism was influenced by CCK-8, while NAD(P)H plays the role of an intermediary here. Coenzymes $NADP^+$/NADPH and NAD^+/NADH are central to the glucose metabolism pathways: NAD^+/NADH is primarily involved in energy-yielding glycolysis and the citric acid cycle, while $NADP^+$/NADPH participates in biosynthetic processes and antioxidant defense, the PPP.[5] These coenzymes shuttle electrons between metabolic reactions, facilitating the transfer of energy and maintaining the redox balance within cells. Carnitine and its derivatives participate in fatty acid metabolism, which involves NAD^+ and NADH in beta-oxidation, as well as $NAD(P)^+$ and NAD(P)H in redox reactions. Carnitine facilitates the transport of fatty acids into the mitochondria, so the decreasing of carnitine can disturb the supply of fatty acids in mitochondria.[6] Furthermore, the enzyme carnitine palmitoyltransferase 1, which plays a central role in fatty acid transport, is regulated by the ratio of NADH to NAD^+ and NADPH to $NADP^+$.[7] The regulation of enzymes involved in carnitine metabolism and fatty acid transport can be influenced by the cellular redox state and the NAD^+/NADH and $NADP^+$/NADPH ratios.

NADPH is a key cofactor required for the enzymatic reduction of oxidized glutathione (GSSG) back to its reduced form, GSH, through the action of the enzyme glutathione reductase.[3] Therefore, we sought to scrutinize the redox state by detecting the GSH changes in MG-63, and as expected, the GSH level was decreased (Figure 1i). The shift toward oxidation disrupts the GSH-GSSG balance, decreasing the cellular GSH pool due to reduced NADPH levels. The alteration in GSH levels impacts the cellular redox state and its defense against oxidants.[8] As a result, if the cells treated with CKK-8 were continued to be applied to other biological evaluations, the result could be misled by this redox state change.

NADPH plays a crucial role as a component of the cellular antioxidant defense system. Utilizing an assay that depletes NADPH could have detrimental effects on studies that rely on these reduced cofactors. For example, the emerging cell death mechanism known as disulfidptosis, which occurs due to inadequate NADPH supply.[9] Consequently, relying on an assay that depletes NADPH may compromise the accuracy of the obtained results associated with disulfidptosis.

Next, we integrated enrichment analysis and pathway topology analysis from the metabolomics data. The metabolism pathways directly related to energy production were significantly influenced (Figure 1f, Figures S4 and S5). The supply of reduced cofactors is ensured by the mitochondrial oxidation of substrates derived from glucose, fatty acids, and amino acids via different metabolic pathways. Therefore, the disruption of homeostasis can cause changes in many cellular functions, such as innate immune responses.[10] It should not be ignored, although the impact could be small. Oxidative phosphorylation is a complex process regulated by interactions between mitochondrial and cytosolic metabolism. These interactions can lead to changes in cellular respiration, influencing cell reactions.[11] As a result, CCK-8 can impact the continued experimental results of the same cells, especially the cell function evaluation relevant to mitochondrial and cytosolic metabolism. Changes were not limited to metabolites directly involved in reduced cofactors; both upstream and downstream metabolites were also altered (Figure 1j).

In our study, we utilized two distinct cell lines, and the findings revealed that CCK-8 can affect the metabolic processes of both cell types. When we compared the fold changes between these two cell types, we observed a significant increase in the level of 6-phosphogluconic acid in both cell lines. However, FBP, ATP, and UTP exhibited changes in opposite directions (see Figure 1k). Notably, the fibroblast cell line displayed greater sensitivity to the depletion of NAD(P)H compared with the osteosarcoma cell line (refer to Figure 1).

The depletion or low regulation of reduced cofactors in cells by CCK-8 can have significant implications of metabolites level. Therefore, the antioxidant defense, biosynthesis, cellular metabolism, detoxification processes, and redox homeostasis are altered.[12] It is essential to note that even when both the experimental and control groups receive the same treatment, the accuracy of results may be compromised by potential synergistic interactions between CCK-8 and the tested drugs or materials. This alteration could significantly influence the results, especially in the color changes, because CCK-8 is a colorimetric method.[13] These interactions have also the potential to induce cellular death and trigger immune-inflammatory responses[14,15] These effects can ultimately impact cell functionality and even the long-term overall cellular state. To conclude, the usage of cells after the CCK-8 kit assay for any subsequent experiment or assay should be approached with caution and consideration of possible implications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.3c00339.

https://doi.org/10.1021/acs.chemrestox.3c00339
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User manual for CCK-8; influence of the CCK-8 assay on cell viability; pathways influenced by CCK-8; Enrichment Network of Metabolite Correlation; materials and methods utilized in the manuscript (PDF)

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Author Contributions

J.F. and K.K. conceptualized and designed the study. J.F. conducted cell experiments, metabolites collection, data analysis, and prepared the manuscript. T.S. and V.J. conducted LIVE/DEAD imaging. A.V. performed LC−MS analysis. K.K. provided supervision, and reviewed and edited the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors acknowledge financial support from the EU research and innovation program under the grant agreement No. 857287 (BBCE − Baltic Biomaterials Centre of Excellence) and the Ministry of Economics Republic of Latvia project "State research project in the field of biomedicine, medical technologies, and pharmacy", Project No. VPP-EM-BIOMEDICĪNA-2022/1-0001.

ABBREVIATIONS

CCK-8, Cell Counting Kit-8; NAD(P)H, nicotinamide adenine dinucleotide (phosphate) hydrogen; GSSG, oxidized glutathione; GSH, glutathione; FBP, fructose-1,6-bisphosphatase; GNG, gluconeogenesis

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