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Influences of Acidic Conditions on Formazan Assay: A Cautionary Note

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Abstract Formazan assay has been used for several decades to evaluate metabolic activity of eukaryotic and prokaryotic cells. In particular, it has been often applied for quantitative assessment of viable cells under acidic circumstances caused by, e.g., ischemia and hypoxia. However, little attention has been paid to the influence of acidic pH on formazan assays. We found that acidic culture conditions significantly affect outcomes of the assays. Absorbance of tetrazolium–formazan decreased in a pH-dependent manner without affecting cell viability. This nonspecific effect was ascribed to influences of acidic pH on the production of formazan. Replacement of culture media to fresh medium at physiologic pH partially overcame this problem. The influence of acidic culture conditions should be carefully considered when formazan assays are used for the assessment of viable cells under various experimental situations.

Keywords Tetrazolium · Formazan · Acidosis · Hypoxia · WST · MTT

Introduction

Tetrazolium salt-based formazan assay is a convenient method for the assessment of viable cell number. The formazan assay is based upon metabolic reduction of tetrazolium salts to colored formazans, and it is mediated by mitochondrial dehydrogenases and other enzymatic systems [1]. Several tetrazolium salts have been used for this purpose. Those

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include 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium (MTS), sodium 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt (WST-1), 4-[3-(4-iodophenyl)-2-(2,4-dinitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt (WST-3), and 4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-l,3-benzene disulfonate sodium salt (WST-8) [1,2]. During the assay, cells are incubated in the presence of tetrazolium salts, which allows for accumulation of formazans. The cultures are then subjected to reading of absorbance using a spectrophotometer. Compared with the prototypic MTT assay and other assays using MTS and XTT, water soluble tetrazolium salt (WST)-based assays (especially the WST-8-based method) achieve better sensitivity, accuracy, and stability [3] (Cell Counting Kit-8, information sheet; Dojindo, Kumamoto, Japan).

The formazan assay has several advantages over conventional approaches for the assessment of viable cells using trypan blue analysis and [^{3}H]-thymidine incorporation. First and foremost, it is simple, easy, and cost-effective. Because of this reason, formazan assays have been widely used for toxicological/pharmacological studies to investigate effects of certain drugs on cellular damage, as well as for physiological/pathological experiments to assess mitogenic or antimitogenic effects of particular factors [1]. However, formazan assays may be affected by some reagents or culture conditions. For example, it is known that reductive agents facilitate production of formazans, leading to significant increases in the background [4]. Agents that trigger reactive oxygen species may also facilitate reduction of some tetrazolium salts [1,4]. Recently, we encountered a problem that cells cultured at high-density exhibit relatively low absorbance in WST assay. We identified that extracellular low pH is a factor responsible for this problem. In the present report, we demonstrate that acidic culture conditions significantly affect outcomes of formazan assays. This artifact is due to influences of acidic pH on the enzymatic reactions to generate formazan. Of note, replacement of culture media to fresh medium at physiologic pH partially overcomes this problem.

Formazan assays are often used for the assessment of cellular damage caused by ischemia and hypoxia, the well-known triggers for acidosis [5,6]. The influence of acidic conditions should be carefully considered when formazan assays are used for the assessment of viable cells.

Materials and Methods

Cells and Reagents

The human mesothelial cell line MeT5A (provided by Dr. Curtis C. Harris, National Institute of Health, Bethesda, MD) [7], the rat mesangial cell line SM43 [8], and the rat renal tubular epithelial cell line NRK-52E (purchased from American Type Culture Collection; Manassas, VA) were used. In general, MeT5A cells were used for studies unless use of other cell types is indicated. Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (pH 7.4; Gibco-BRL, Gaithersburg, MD) supplemented with 1% fetal bovine serum was generally used for studies. In some experiments, DMEM/Ham's F-12 with L-glutamine and phenol red (pH 7.8; Wako, Osaka, Japan) was also used. Media at acidic pH were prepared by adding with 2-morpholinoethanesulfonic acid monohydrate (MES; Dojindo, Kumamoto, Japan). Acetic acid (Wako) and hydrochloric acid (Wako) were also used in some cases.

Cell Counting

The number of viable cells was assessed by trypan blue exclusion. In brief, cells in 24- or 96-well plates were trypsinized, centrifuged, and suspended in small volumes of medium. The cell suspensions were added with the same volume of trypan blue solution and subjected to counting of viable cells using a hemocytometer.

Formazan Assays

WST assay was performed using Cell Counting Kit-8 (Dojindo) following the protocol provided by the manufacturer. In brief, cells in 96-well plates were incubated for 1 h in media containing WST-8 (10% Cell Counting Kit-8 solution), and absorbance was read at 450 nm by a spectrophotometer. The background absorbance was also measured at 650 nm. The absorbance at 450 nm was subtracted by that at 650 nm and subjected to analysis, following the protocol of Cell Counting Kit-8. Of note, the background absorbance was not altered under acidic conditions. MTT assay was performed as follows: Cells in 96-well plates were first incubated for 3 h in culture medium containing 250 μg/ml MTT (Dojindo). After termination of the reaction by adding 20% SDS (*w*/*v*) and 50% dimethyl formamide (*v*/*v*), the absorbance was read at 570 nm.

Statistical Analysis

All assays were performed in quadruplicate, and data were expressed as means ± SE. Statistical analysis was performed using the nonparametric Mann–Whitney *U* test to compare data in different groups. *P* value <0.05 was considered to indicate a statistically significant difference.

Results and Discussion

MeT5A cells were cultured at physiologic pH (7.4) or acidic pH (6.2; adjusted by MES) for 1 h and subjected to trypan blue analysis and WST assay. As predicted, the number of viable cells was not altered by the treatment with acid, when examined by trypan blue exclusion (Fig. 1a). However, the level of absorbance was significantly lower under the acidic condition (Fig. 1b). This phenomenon was observed similarly when pH was adjusted to 6.2 by acetic acid (Fig. 1c and d) or hydrochloric acid (not shown). Of note, this effect was reproducible even when different culture media (DMEM/Ham's F-12 with L-glutamine/phenol red and phosphate-buffered saline) were used (Supplemental Figs. 1, 2). Of note, blank absorbance of medium alone was not decreased under the acidic condition (Supplemental Fig. 3). To further confirm that acidic conditions affect the outcome of the assay, cells were exposed to serial acidic pH and subjected to WST assay. As shown in Fig. 1e, absorbance decreased under the acidic conditions in a pH-dependent manner. Significant reduction was observed at pH≤7.0.

In the experiments described above, we used WST-8-based assay. To confirm that other formazan assays using different tetrazolium salts are also influenced by acidic pH, we performed experiments using MTT. Cells were cultured under physiologic or acidic pH for 3 h in the presence of MTT and subjected to analyses. Consistent with the results shown in Fig. 1b and d, the level of absorbance was significantly lower under the acidic condition (Fig. 1g), although the number of viable cells was not different when examined by trypan

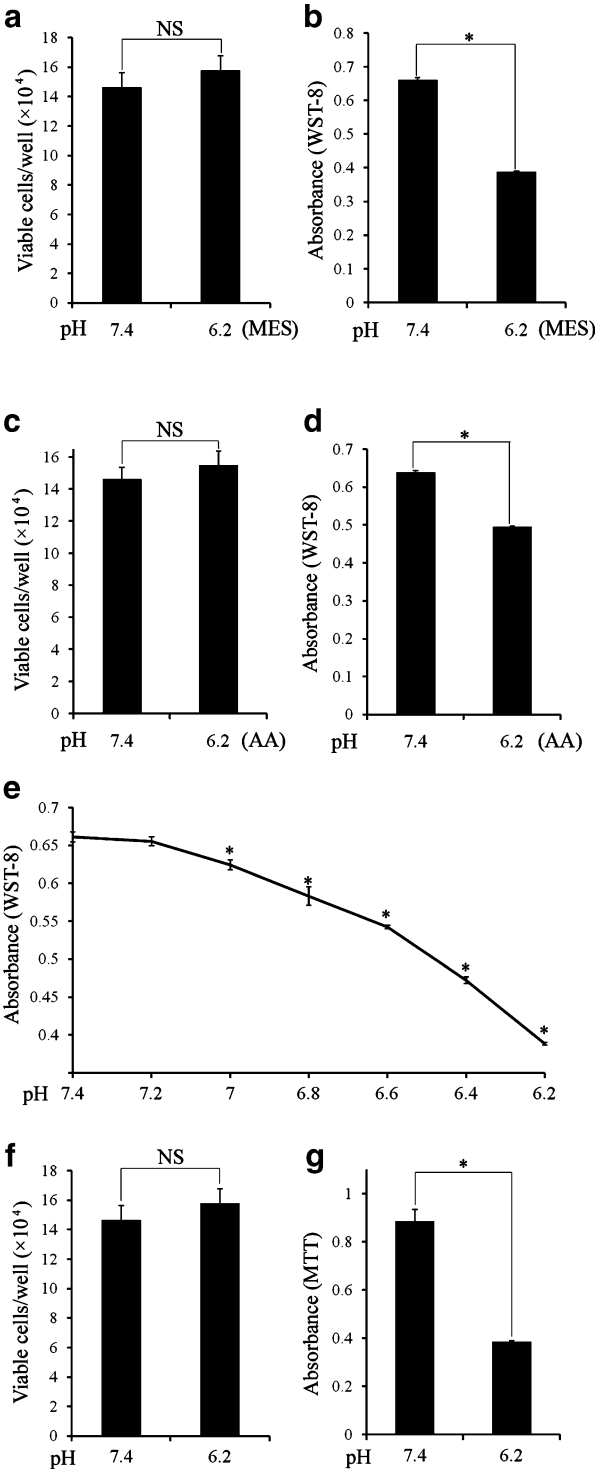

Fig. 1 Influence of acidic pH on WST and MTT assays. **a**–**d** MeT5A cells were seeded in 24-well plates (**a**, **c**) or 96-well plates (**b**, **d**), incubated overnight, exposed to medium at pH 7.4 or 6.2 (adjusted by *MES* (**a**, **b**) or acetic acid (indicated as "*AA*") (**c**, **d**)) for 1 h, and subjected to cell counting (**a**, **c**) and WST assay (**b**, **d**). Assays were performed in quadruplicate, and data are expressed as means ± SE. *Asterisks* indicate statistically significant differences ($p<0.05$). *NS* not statistically significant. **e** Cells were incubated for 1 h at indicated pH (adjusted by MES), and WST assay was performed. **f**, **g** Cells were incubated for 1 h at pH 7.4 or 6.2 and subjected to trypan blue analysis (**f**) and MTT assay (**g**)

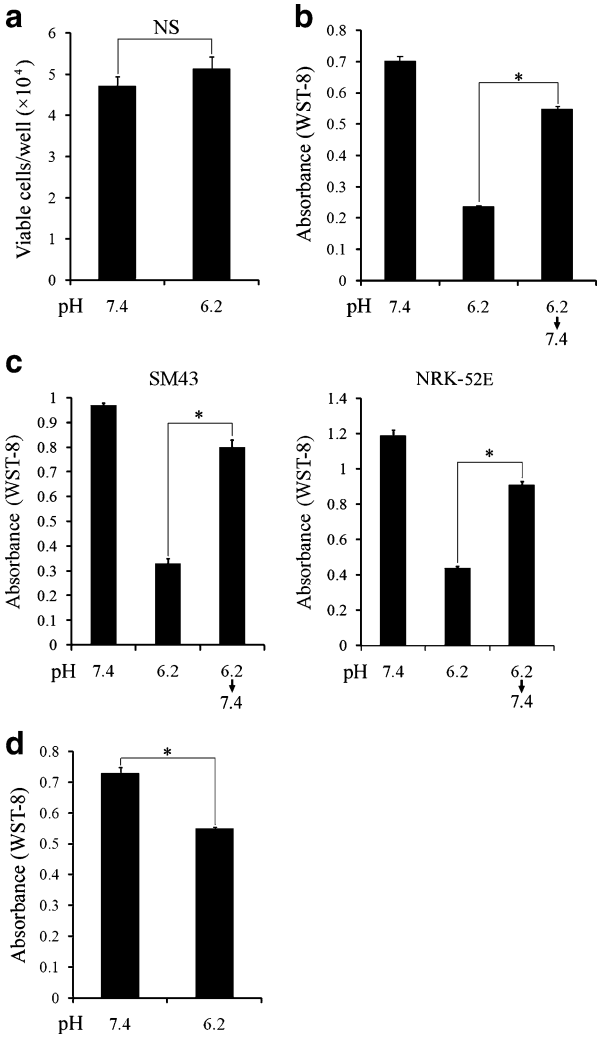

Fig. 2 Mechanisms underlying decreases in absorbance by acidic conditions. **a**, **b** MeT5A cells were seeded into 96-well plates (**b**) and incubated for 5–6 h at pH 7.4 or 6.2 (adjusted by MES). After the incubation, the culture media were replaced with or without physiologic medium (pH 7.4), further incubated with WST-8 for 1 h and subjected to cell counting (**a**) and WST assay (**b**). **c** Similar experiments were performed using SM43 cells and NRK-52E cells. **d** Cells were incubated at pH 7.4 or 6.2 for 6 h. After the incubation, the culture medium was replaced with fresh medium at pH 7.4 and subjected to WST assay

blue analysis (Fig. 1f). These results suggest that acidic pH affects the outcome of formazan assays regardless of tetrazolium salts used.

To exclude a possibility that acidic conditions affect stability of formazan, WST-8-formazan produced by cells was treated with MES for 1 h and subjected to analysis. As shown in Supplemental Fig. 4, significant reduction in absorbance was not observed under the acidic condition.

If acidic pH significantly affects the outcome of the assays, replacement of culture media prior to incubation with tetrazolium salts may overcome the problem. To examine this

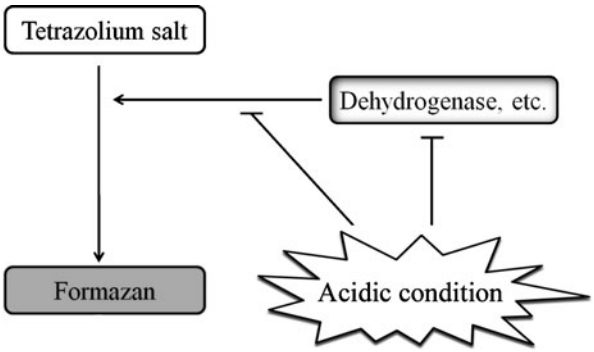

Fig. 3 Influences of acidic conditions on formazan assay. Acidic condition reduces absorbance through interfering with formazan-producing enzymes such as dehydrogenase and enzymatic reaction

possibility, MeT5A cells were first incubated for 6 h under the acidic condition. The cells were then exposed to physiologic or acidic medium containing WST-8 and subjected to analyses. As shown in Fig. 2a, incubation of the cells at low pH for 6 h did not alter the number of viable cells. Acidic pH caused a significant reduction in the level of absorbance. Replacement of culture medium and incubation with WST-8 at pH 7.4 reversed this suppressive effect (Fig. 2b), indicating that acidic pH affected enzyme reaction responsible for the production of formazan. To further confirm this result, SM43 cells and NRK-52E cells were subjected to the analysis using the same experimental protocol. Consistent with the result in MeT5A cells, acidic condition caused significant reductions in the level of absorbance in both cell types, but incubation with WST-8 at physiologic pH reversed this suppressive effect (Fig. 2c).

We examined mechanisms underlying the effect of acidic conditions on formazan assay. Cells were first incubated under physiologic or acidic pH for 6 h. After the incubation, the culture medium was replaced with fresh medium at pH 7.4 and subjected to WST assay. Under this experimental setting, the absorbance was still lower in the acidic group, indicating a possibility that the acidic condition may modestly deteriorate dehydrogenases or other responsible enzymes during the initial incubation (Fig. 2d). The influences of acidic pH on formazan assays are schematically presented in Fig. 3.

Acidosis is often caused in cell cultures under various experimental situations; e.g., use of high-cell density, long-term incubation, exposure to hypoxia, and addition of acidic reagents to examine effects of acidic environments on cell survival, proliferation, and gene expression. In the experiments using hypoxia/reoxygenation and extracellular acidosis, formazan assays have been often used to evaluate cell proliferation and/or cellular injury. However, little attention has been paid to the influences of acidic pH on formazan assays. For example, some groups investigated effects of citric acid and associated extracellular acidosis on the viability of human gingival fibroblasts using MTT assay. The authors concluded; (1) culture medium with citric acid led to extracellular acidosis and cell death and (2) the citric-acid-induced death was prevented by adjusting the low pH to physiologic pH [9]. Other groups investigated changes of vitreous pH under acute glaucoma status in rabbits. The authors found that vitreous pH decreased under acute glaucoma and that the acidic pH caused retinal cell death when evaluated by MTT assay [10]. Ischemic injury is one of major interests in the biomedical research field, and a number of studies have been conducted to elucidate mechanisms involved in this pathological process [11]. “Oxygen/glucose deprivation followed by reoxygenation” is commonly used as an in vitro model of

the ischemic injury. Some reports showed that acidosis during reoxygenation enhanced cellular injury using MTT assay [12]. These cases are only the tip of the iceberg. Database search for the literature showed that formazan assays have been used in more than 600 reports focusing on the ischemic/hypoxic cell injury.

We suggest that, under acidic situations, especially under ischemic and/or hypoxic conditions, use of formazan assays should be careful, and direct effects of acidosis on the assays must be considered. It is also worthwhile to note that replacement of culture media prior to formazan assays can substantially overcome this problem.

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References

1. Berridge, M. V., Herst, P. M., & Tan, A. S. (2005). *Biotechnology Annual Review, 11*, 127–152.
2. Ishiyama, M., Miyazono, Y., & Sasamoto, K. (1997). *Talanta, 44*, 1299–1305.
3. Tominaga, H., Ishiyama, M., Ohseto, F., Sasamoto, K., Hamamoto, T., Suzuki, K., et al. (1999). *Analytical Communications, 36*, 47–50.
4. Kim, H., Yoon, S. C., Lee, T. Y., & Jeong, D. (2009). *Toxicology Letters, 184*, 13–17.
5. Hochachka, P. W., & Mommsen, T. P. (1983). *Science, 219*, 1391–1397.
6. Zager, R. A., Schimpf, B. A., & Gmur, D. J. (1993). *Circulation Research, 72*, 837–846.
7. Duncan, E. L., Whitaker, N. J., Moy, E. L., & Reddel, R. R. (1993). *Experimental Cell Research, 205*, 337–344.
8. Kitamura, M., Taylor, S., Unwin, R., Burton, S., Shimizu, F., & Fine, L. G. (1994). *Journal of Clinical Investigation, 94*, 497–505.
9. Lan, W. C., Lan, W. H., Chan, C. P., Hsieh, C. C., Chang, M. C., & Jeng, J. H. (1999). *Australian Dental Journal, 44*, 123–130.
10. Lu, D. W., Chang, C. J., & Wu, J. N. (2001). *Journal of Ocular Pharmacology and Therapeutics, 17*, 343–350.
11. Li, C., & Jackson, R. M. (2002). *American Journal of Physiology Cell Physiology, 282*, C227–C241.
12. Almaas, R., Pytte, M., Lindstad, J. K., Wright, M., Saugstad, O. D., Pleasure, D., et al. (2003). *Journal of Neurochemistry, 84*, 1018–1027.