

Short Research Communication

Long Term and Standard Incubations of WST-I Reagent Reflect the Same Inhibitory Trend of Cell Viability in Rat Airway Smooth Muscle Cells

Lei-Miao Yin, Yin Wei, Yu Wang, Yu-Dong Xu and Yong-Qing Yang[✉]

Laboratory of Molecular Biology, Shanghai Research Institute of Acupuncture and Meridian, Yue Yang Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200030, China.

✉ Corresponding author: Fax: +86-21-54592134; E-mail: yyq@shutcm.edu.cn.

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Abstract

The WST-I assay is an efficient test for cell viability measurement and the standard incubation time is 2h. In order to test if one-time addition of WST-I reagent can reflect the relative cell viability trend of the testing agents at different time points, the effects of 2h standard incubation time and long term incubation time (2h+24h, 2h+48h) of WST-I were compared in the rat airway smooth muscle cells (ASM cells) after adding of the testing protein MRP-14. Our study demonstrated that the effect of different dosages of the protein after 2h WST-I incubation on ASM cells showed a tendency of inhibition and achieved the maximal inhibition effect at 72h. The relative cell viability trend of the 2h+24h group was the same to that of the 2h WST-I incubation, which means that 24h prolonged incubation time of WST-I reagent could still reflect the relative cell viability trend. In conclusion, the study suggested that the WST-I is a proper candidate reagent for continuous monitiation of cell viability.

Key words: WST-1, Cell viability, Rat airway smooth muscle cells.

Introduction

The cell viability measurement is one of the most fundamental tests in different forms of cell culture, which tests the number of healthy cells in a sample. There are more than 10 types of methods for investigating cellular viability[1], such as the trypan blue dye exclusion assay, the resazurin-based methods (alamar blue), the measurement of up-taking radioactive labeled ³H-thymidine into cellular DNA, and the reduction of different kinds of tetrazolium salts, etc[2]. The trypan blue dye exclusion assay is the earliest method for assessing cell viability, which is still used today due to the simplicity of both operations and reagents. The resazurin-based methods can be measured by both colourimetry and fluorimetry, which was reduced to a pink fluorescent dye in the medium by cell activity[3]. Measuring the up-taking of ³H-thymidine is another classic method, although it

involves the application of radioactive products and special facilities. The cell viability status can also be simply reflected by the metabolic activity, which is capable of being estimated quantitatively by adding tetrazolium salts to cells[4].

The colorimetric methods have been widely used nowadays, such as MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), XTT (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-carboxanilide-2H-tetrazolium, monosodium salt), MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt), WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt), etc. The MTT assay is one of the the most commonly used ways to detect the level of cell methabolism, which produces an intense color upon cellular reduc-

tion due to the formation of water insoluble formazan[5]. However, the MTT method needs to break the cells and dissolve the precipitates. The assay takes approximately 44-52h and should be measured at 570nm[6, 7]. As the second generation tetrazolium dyes, the XTT could be transformed to a water-soluble formazan derivative which absorbs light at 430-490 nm in the presence of active mitochondria[8]. The absorbance range of another tetrazolium dye MTS was 515-580 nm and the color development speed is fast[9].

The WST-1 reagent could be reduced to highly water soluble formazan by cellular dehydrogenases in the presence of intermediate electron acceptor, such as mPMS (1-methoxy-5-methyl-phenazinium methyl sulfate)[10]. The mechanism is shown in the Figure 1. The formazan produced by WST-1 is more soluble than that of XTT and MTS, which leads to a wider linear range and higher sensitivity. Though it is reported that WST-1 derivant could still be metabolized to the formazan product after 48h incubation[11], the standard incubation time of WST-1 time is 2h[6]. Whether one-time addition of WST-1 can reflect the effect of the testing agents at different time points on the trend of relative cell viability is still unclear.

In order to improve efficiency and simplify manipulation, the effects of 2h standard incubation time and long term incubation (2h+24h, 2h+48h) of WST-1 in the rat airway smooth muscle cells were compared after adding of the testing protein MRP-14. We want to investigate if the long term incubation of WST-1 reagent can reflect the trend of relative cell viability at 24h, 48h, and 72h, which could be used potentially in the continuous monition of cell viability.

Method

Chemicals and reagents

All chemicals were analytical grade. WST-1 reagents (Beyotime), Dulbecco's modified eagle's medium (DMEM, Hyclone), fetal bovine serum (FBS, Gibco), phosphate buffered saline (PBS, Hyclone), 0.25% trypsin-EDTA solution (Gibco), penicillin-streptomycin solution (10,000 U/ml penicillin and 10,000 µg/ml streptomycin, Hyclone), Spectrophotometer (Bio-tek). The 100 cm² cell culture dishes and 96-well plates were purchased from Corning. The MRP-14 recombinant protein was purified by our previous study[12], which is a 13 kDa soluble protein and has two Ca²⁺-binding EF-hand motifs[13].

Isolation and culture of rat airway smooth muscle (ASM) cells

Rat trachea was placed into the sterile, ice-cold,

HPPS solution (NaCl 130.0 mM, KCl 5.0 mM, MgCl₂·6H₂O 1.2 mM, HEPES 10.0 mM, and glucose 10.0 mM, pH=7.4). The surrounding redundant tissue was dissected from cartilage and washed. The trachea was then cutted into pieces and digested for 30 min at 37°C in HPPS solution containing 2.0 mg/ml collagenase IV and 0.05% elastase. Enzyme digests were subsequently centrifuged at 1000 rpm for 5 min, and the pellet was resuspended and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, at 37°C in a 5% CO₂ atmosphere. The culture medium was renewed every 2-3 days and experiments were performed with cells on passages 3-10. The ASM cells were confirmed by the light microscope and the immunofluorescent test confirmed that more than 95% of primary culture cells expressed contractile protein SM α-actin, which was the specific marker of airway smooth muscle.

Incubation of WST-1

ASM cells were harvested by trypsinization and were resuspended in DMEM containing 10% FBS. The cells were then plated at a density of 5000 cells per well into the 96-well plates and incubated overnight. The MRP-14 recombinant protein was given at the concentration of 1, 10, 50, 100, 200, 400, 800 ng/ml (final concentration) into totally three 96-well plates and incubated for 24h, 48h, 72h, respectively. Five replicates were used at each concentration and the PBS buffer was added in the control group. After adding the WST-1 into the first plate, the reagent was kept in the 96-well plate and incubated for 2h (standard incubation time), 2h+24h and 2h+48h (long term incubation). The last two measurements were taken at the same time with the 2h incubation of WST-1 at 48h and 72h. The detailed protocol was shown in the Figure 2. The absorbance at 450 nm was monitored and the reference wavelength was set at 630 nm. The relative cell viability percentage in each group was calculated by comparison to that of the control group.

Statistics

All data are expressed as the mean ± SD. Statistical significance between different groups in the WST-1 incubation study was calculated using One-way ANOVA followed by LSD posthoc test. P values lower than 0.05 were considered significant.

Result

According to the data of 2h incubation of WST-1, the different dosages of MRP-14 recombinant protein showed a tendency of inhibition and achieved the maximal inhibition effect at 72h. At 24h, there was no significant difference between different groups

($F=1.402$, $P>0.05$), though the dosages of 50, 100, and 800 ng/ml proteins had showed an inhibition tendency on ASM cells (Figure 3A). At 48h, there was a significant difference between different groups ($F=9.859$, $P<0.05$) and the dosages of 400 and 800 ng/ml proteins significantly inhibited the viability of ASM cells ($P<0.05$ vs dosage of 1 ng/ml, Figure 2B). The relative cell viability trend of the 2h+24h group was the same to that of the 2h WST-1 incubation (Figure 3C). There was a significant difference between different groups ($F=15.766$, $P<0.05$) after 2h+24h incubation and the effect of the dosages of 200, 400 and 800 ng/ml protein were significantly different from that of the 1 ng/ml dosage ($P<0.05$).

At 72h, there was a significant difference between different groups ($F=9.533$, $P<0.05$) and the dosages of 50, 100, 200, 400, and 800 ng/ml proteins significantly inhibited the viability of ASM cells ($P<0.05$ vs dosage of 1 ng/ml, Figure 3D). The 800 ng/ml intervention achieved the maximum effect. Although the relative cell viability trend of the 2h+48h group is different from that of the 2h WST-1 incubation (Figure 3E), there was a significant difference between different groups ($F=2.504$, $P<0.05$) after 2h+48h incubation and the effect of the dosage of 800 ng/ml protein was significantly different from that of the 1 ng/ml dosage ($P<0.05$).

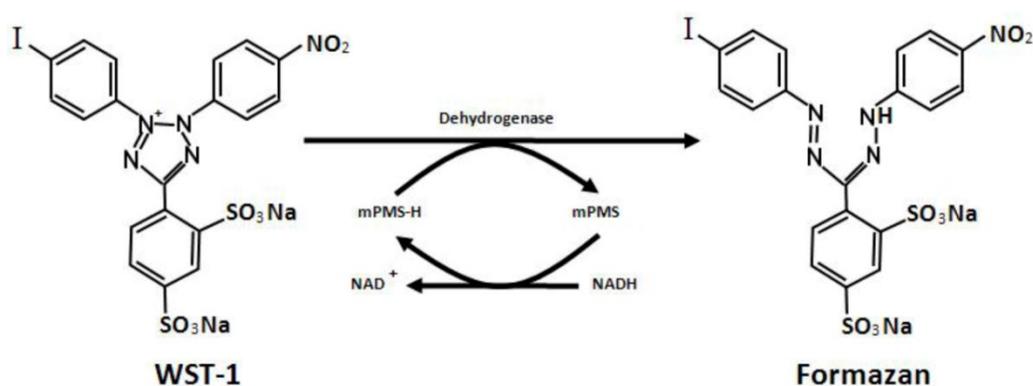


Figure 1 Schematic mechanism of the WST-1 reduction. With intermediate electron acceptor (such as mPMS), the WST-1 reagent could be reduced to highly water soluble formazan by cellular dehydrogenases. Abbreviation: mPMS: 1-methoxy-5-methyl-phenazinium methyl sulfate; NADH: Nicotinamide adenine dinucleotide.

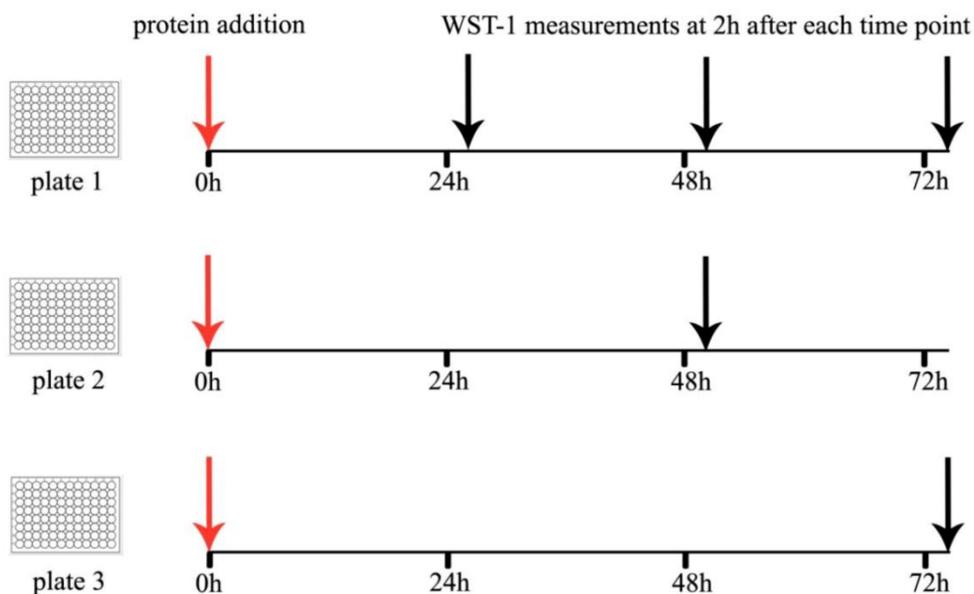


Figure 2 Schematic of the WST-1 incubation study. The MRP-14 recombinant protein was added into totally three 96-well plates, which were measured at 24h, 48h, 72h, respectively. After adding the WST-1 into the first plate, the reagent was kept in the 96-well plate and incubated for 2h, 2h+24h and 2h+48h. The last two measurements were taken at the same time with the 2h incubation of WST-1 at 48h and 72h.

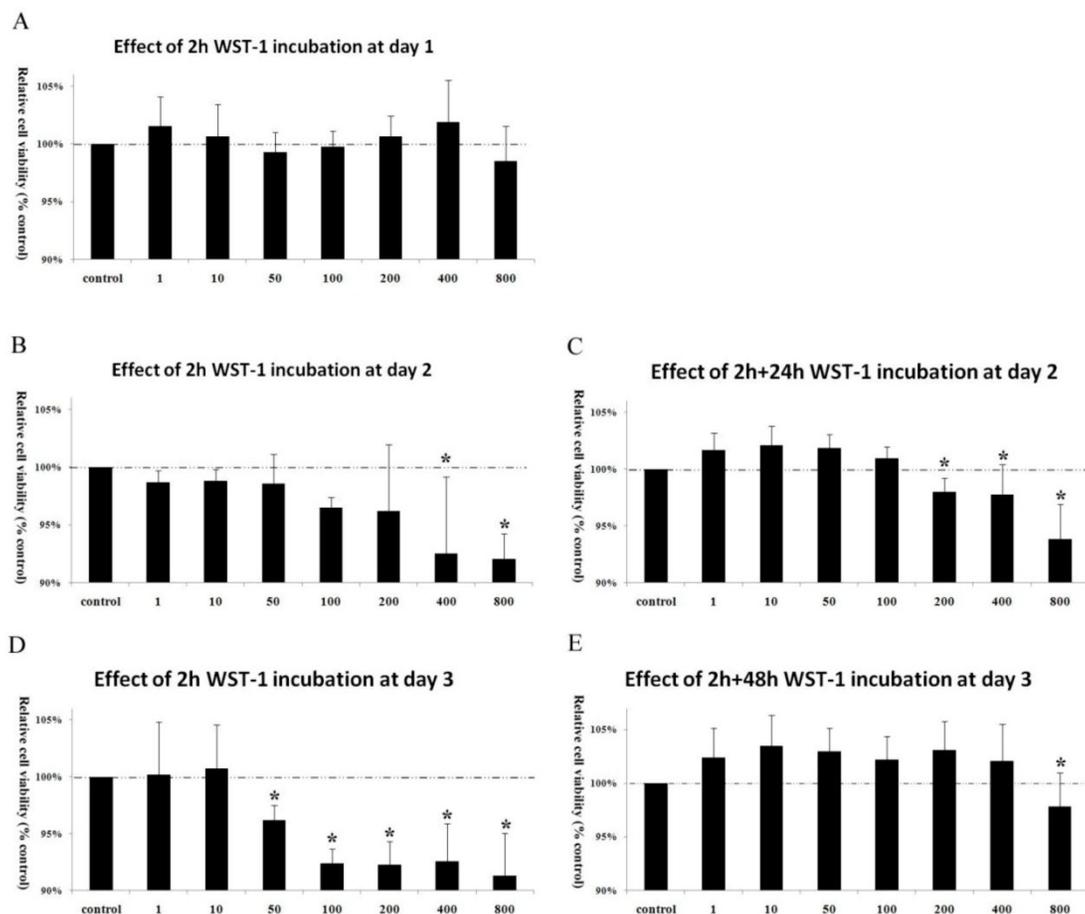


Figure 3 Effect of WST-1 incubation at different time points. A) The effect of different dosages of protein after 2h WST-1 incubation on the rat airway smooth muscle (ASM) cells at 24h. B) The effect of different dosages of protein after 2h WST-1 incubation on ASM cells at 48h. The dosages of 400 and 800 ng/ml proteins significantly inhibited the viability of ASM cells ($P < 0.05$ vs dosage of 1 ng/ml). C) The effect of different dosages of protein after 2h+24h WST-1 incubation on ASM cells at 48h. The dosages of 200, 400 and 800 ng/ml protein were significantly different from that of the 1 ng/ml dosage ($P < 0.05$). D) The effect of different dosages of protein after 2h WST-1 incubation on ASM cells at 72h. The dosages of 50, 100, 200, 400, and 800 ng/ml proteins significantly inhibited the viability of ASM cells ($P < 0.05$ vs dosage of 1 ng/ml). E) The effect of different dosages of protein after 2h+48h WST-1 incubation on ASM cells at 72h. The dosage of 800 ng/ml protein was significantly different from that of the 1 ng/ml ($P < 0.05$). All data are expressed as the mean \pm SD, $n=5$.

Discussion

The inhibitory effect of MRP-14 on cell viability by using WST-1 is consistent with previous researches that showed the protein was related with the regulation of cellular processes, such as metabolism and cell cycle progression[14-15]. In the present study, we found that the 24h prolonged incubation time of WST-1 reagent could still reflect the relative cell viability trend, which could be used potentially to continuous detection.

The investigation of cellular viability is a fundamental technique for the assessment of biological reaction of cell to outside stimuli. The WST-1 assay permits the measurement of a large number of samples without generating radioactive waste and exhibited higher precision, which now plays a important

role in the investigation of cellular viability[16]. The assay steps of WST-1 are simple and the test time is short. It is reported that the WST-1 test was efficient and fast in screening for radiationsensitive cell lines in 120 cancer patients[17]. Besides, the WST-1 assay could also be used for 3h rapid confirmation of toxigenic *Bacillus* species in foods[6]. However, WST-1 assay was usually performed once at a single time point. No previous study has traced the long term incubation at different time point after the addition of WST-1. Our study demonstrated that the relative cell viability trend of the 2h+24h group was the same to that of the 2h WST-1 incubation, although the original OD values of the WST-1 test at the 2h+24h was higher. The study suggested that the WST-1 reagent is proper for continuous monition of cell viability.

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Competing Interests

The authors have declared that no competing interest exists.

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