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Verification and Improvement of the MTT Method for in vitro HSS Bioactivity Activity Determination

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Abstract: To optimize the experimental conditions of MTT colorimetric assay for HSS bioactivity in vitro, we studied the optimal combination of the major conditions of the MTT assay by orthogonal test and other experiments, and compared HSS bioactivity in vitro measured by the improved MTT protocol and published MTT assay at serial protein doses. Results showed that the absorbance value (A value) of the MTT assay directly correlated with the number of human hepatoma cell lines SMMC7721. The result of orthogonal test was the number of 5×104 SMMC7721 cells/ml, culture period 6 h before adding HSS, concentration of HSS 100 μg/ml, incubation time with HSS 36 h. Additionally, several experiments demonstrated the optimal combination of other conditions was 50 μg MTT, incubation time for MTT 6 h, DMSO was used to dissolve the MTT formazan crystals and measured with ELISA scanner at 570 nm. The result of determining HSS bio-activity in vitro by optimized MTT protocol showed that sHSS bio-activity increased with the growth of protein dose, but decreased when it beyond a certain dose. The optimized MTT protocol was a sensitive, convenient and stable quantitative method to evaluate HSS bio-activity.

Key words: HSS (Hepatic Stimulator Substance); MTT colorimetric assay; Bio-activity

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Hepatic stimulator substance (HSS) is a polypeptide known as a liver growth stimulator derived from the hepatocyte cytosol of mammalian liver. In our previous report[1,2], we described the purification, physicochemical characteristics, N-terminal sequence, and bioactivity of HSS which was derived from a kind of marine organism-shark liver.

In the original studies of HSS, the incorporation of [3H] thymidine (3H-TdR method) into liver DNA was generally used to evaluate its bioactivity, but this method has the definite drawback of requiring radioisotopes[3]. In 1991, enzyme-linked immunosorbent assay (ELISA) was applied to measure the number of viable[4] cells, but it is necessary to wash the cultured cells and these steps increase the processing time .The colorimetric assay, which is based on the conversion of the yellow tetrazolium salt 3-(4, 5-dimethy1-2-thiazolyl)-2, 5-dipheny1-2H-tetrazolium bromide (MTT) to the blue formazan derivative by mitochondrial enzymes in viablecells, has been proven to be valuable for detecting the bioactivity of, for example, interleukin[5], colony stim-ulating factor[6], hepatocyte growth factor[7], hepatic stimulator substance[2], uroacitide[8], and so on[9, 10]. In the paper reported here we have examined MTT assay in detail and explored the critical laboratory parameters involved in the technique. Optimizationand improvement were also attempted and direct comparison was made with published method.

1Materials and Methods

1.1 Materials

1.1.1Cell lines and medium

RPMI 1640 medium(Hyclon, USA), supplemented with 100 U/ml of streptomycin, 100U/ml of penicillin, 2.2 mg/ml $NaHCO_3$, and 10% of inactivated fetal calf

serum (FCS; Sijiqing Biotect, China) was used as a basal medium. Human hepatoma cell lines SMMC 7721 was stored by our laboratory, and cultured in the basal medium at 37 ℃ in a humidified chamber with 5 %CO_2.

1.1.2sHSS

Shark hepatic stimulator substance(sHSS) was extracted and purified by our laboratory. Chemicals 3, (4,5-dimethylthiazol-2-yl)2, (5-diphenyltetrazolium bromide) (MTT; Amresco, USA) was dissolved at different concentration in PBS pH 7.2), sterilized by filtration and stored at 4 ℃ in a darkened bottle. Dimethyl sulfoxide (DMSO, analytical grade) was obtained from Lingfeng chemical reagent company of Shanghai. SDS (Practical grade) was pruchased from Amresco and dissolved in the solution of 0.01 mol/ L HCl. Isopropanol (analytical grade) was obtained from Nanjing chemical reagent No.1 factory.

1.2 Methodes

1.2.1 MTT assay

200μl of cell suspension were introduced into each well of 96-microwell culture plate. Following incubation for 0h ～ 12h, 200 μl of HSS sample were added to individual well. The cells were continu
ously exposed to the drugs during 12h ～ 60h incubation. 100μl of the MTT solution were added to each well and incubated for 6h .These crystals were dissolved in 100μl of different solvent by agitating on a plate shaker for 10min ～ 24h. The absorbance value of the wells was measured at wavelength 400-700 nm. Control wells containing cells and medium were tested at the same time. Wells containing medium only were used to blank the ELISA scanner.

1.2.2 Orthogonal test

The effectiveness of MTT as-say for determining HSS bioactivity was affected by several factors, and cell density, HSS concentration, culture period before adding HSS, and duration of incubation atrer adding HSS are among the major factors. In order to optimize MTT assay, an orthogonal test with 4 factors and 3 levels was designed (Table 1) , Table 2 showed the L9 (34) orthogonal table. HSS bio-activity was determined as A (HSS)/A(control).

	A	B	C	D
Factors	Cell density	Concentration of HSS	Culture period befor	Duration of incubation
	(×10^4cells/ ml)	(μg/ml)	adding HSS (h)	whth HSS (h)
Level 1	20	100	0	12
Level 2	10	50	6	36
Level 3	5	25	12	60

Table 1. The factors and levels in MTT orthogonal test

2Results

2.1 Cell number

Figure 1 shows a linear correlation between the number of human hepatoma cell line SMMC 7721 and the A value over a wide range from 5 ×103 cells/well to 8 ×104 cells/well. So the MTT assay is suitable for measuring cell number of SMMC 7721 within this range. Cell number below 5×103 cells/well and above 8 ×104 cells/well yielded a signal which was not reproducible.

2.2 Dose of MTT

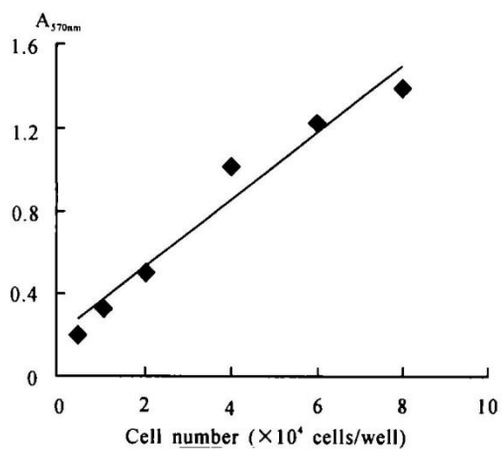

Figure 1. The linear regression analysis.

Each point shows the mean of four replicates (standard deviations are all<10 %, not shown). (y =0.1610x +0.1923, r=0 .98, P<0.01).

From Figure 2 it can be seen that there is an obvious increase in the absorbance signal providing the dose of MTT below 50μg, and then the absorbance curve drops continously. It was therefore decided to use 50μg MTT for incubation of 6h.

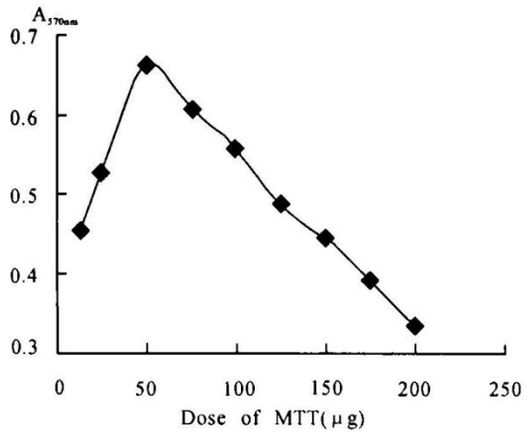

Figure 2. The A value of formazan with increasing doses of MTT.

The data represent the means of four replicates, standard deviations ranging from 1 to 10%.

2.3 Orthogonal test

The result of orthogonal test (Table 2) shows that the effect of each factor on evaluating in vitro HSS bio-activity in turn: B >D ≈AC. From analysis of variance table (Table 3) it can be seen that A, B, D are significant factors of MTT colorimetric assay for evaluating HSS bio-activity, but factor C is insignificant .The optimized experimental conditions are A3B1C2D2, 5×104 cells/ml, culture period befor adding HSS 6h, 100 μg/ml HSS for 36h .Using the optimized conditions A3B1C2, we tested different culture time with HSS for 24h, 30h, 36h, 42h, 48h, respectively, and found that incubation with HSS for 30h ～ 42h yielded similar results (data not shown).

Table 2. L9(34) orthogonal table

Factors	A	B	C	D	A (HSS)/A (control) *
NO 1	1	1	1	1	1.1558
NO 2	1	2	2	2	1.1947
NO 3	1	3	3	3	0.9762
NO 4	2	1	2	3	1.2281
NO 5	2	2	3	1	1.1689
NO 6	2	3	1	2	1.1672
NO 7	3	1	3	2	1.3977
NO 8	3	2	1	3	1.1404
NO 9	3	3	2	1	1.1982
K1	1.1089	1.2605	1.1545	1.1743	—
K2	1.1881	1.1680	1.2027	1.2532	—
K3	1.2452	1.1139	1.1809	1.1149	—
R	0.1365	0.1466	0.0525	0.1383	—

* The data represent the means of eight replicates, standard deviations ranging from 1 to 10%.(not shown)

Table 3. Analysis of variance table

Source of variance	Sum of deviation square	Degrees of freedom	Evaluated variance	F value	Fα
A	0 .0282	2	0.0141	5.42	F0.05(2, 7) =4 .47
B	0 .0330	2	0.0165	6.35	F0.05(2, 7) =4 .47
D	0 .0289	2	0.0145	5.58	F0.05(2, 7) =4 .47
Error *	0 .0185	7	0.0026		

* Sc was added to error term in order to enhance the reliability of F-test.

Such experiments of optimum conditions were repeated with independently prepared HSS samples on three different batches. Each of three results (A (HSS)/A(control) is 1.4255, 1.5617, 1.4574, respectively) is significantly higher than anyone of the or shogunal table.

2.4 Solvent and absorbance spectrum

4 potential solvent of MTT formazan were examined in accordance with published methods[11-14]: DMSO (1987, Carmichael), DMSO: Ethanol (1993, Sladowski), 10%SDS in 0.01mol/L HCl (1986, Ta-da) and isopropanol-0 .04mol/L HCl (1983, Masmann) (Table 4).The absorbance value at 570 nm were measured during 10min ～ 24h following the addition of solvent. 10% solution of the detergent SDS was added on top of the wells unnecessary for discarding the culture supernatant, but this was followed by overnight incubation at 37 ℃. Microscopic examination revealed the presence of small dye grains. Isopropanol caused protein precipitation formed in FCS-containing culture media and significant light scattering that interfered with the measurement of the absorbance value .The result form the experiment showed that the absorbance value of DMSO alone was approximately twice as high as that of the mixture of DMSO-ethanol. (data not shown)

On the whole, DMSO is thought to be the best to dissolve formazan crystals produced by SMMC 7721 cells and unconverted MTT and remnant medium must be removed prior to the addition of DMSO .The absorbance spectrum of formazan crystals in DMSO is shown in Figure 3 from 400nm ～ 700nm at in tervals of 20 nm. The absorbance peak of the dissolved formazan is 560 nm.

Table 4. Comparison of solvent on MTT formazan

Solvent	Solubility	Time for solubilization	Volatility	Causticity
DMSO	+++	10 min	+	-
DMSO :Ethanol 1 1	++	10 min	+	-
10%sds-0 .01mol/ L HCl	+	12h	-	+

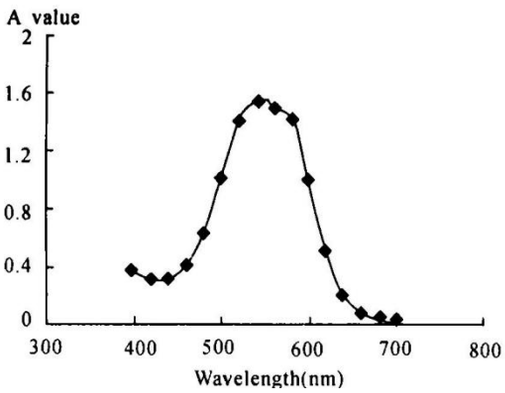

Figure 3. The absorbance spectrum of formazan dissolved in DMSO.

SMMC 7721 cells(2 ×104 ceels/ well) were seeded in RPMI 1640 -10%FCS, and after incubation of 6h, 100μl of 0 .5mg/ ml MTT were added to each well for further culture of 6h .The absorbance value was measured from 400nm ～ 700nm at intervals of 20nm after formazan was solubilized by DM-SO for 10 min.

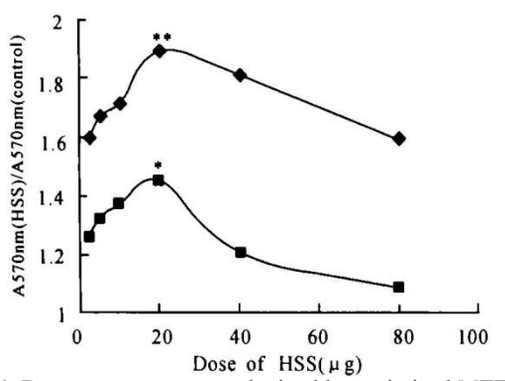

Figure 4. Dose-response curves obtained by optimized MTT protocol (◆-◆) and published MTT assay (■-■) .

Results are expressed as the ration of the mean A570nm in HSS-treated wells to that in control wells(without HSS). Each value represents the mean of four replicates, and the mean of standard deviations in the optimized MTT protocol(4 .6%) is comparable to that observed in the published MTT assay (3.5%) (not shown). Those values that were significantly different from controls are noted. ＊＊ P<0.001, ＊ P<0.01

2.5 Comparison of HSS bio-activity measured by the optimized MTT protocol and published MTT assay

Figgure 4 shows the dose-response curves of the same HSS sample by the optimized MTT protocol and the published MTT assay[2] using the same preparations of SMMC 7721 cells .The direct comparison of the results obtained by the two methods demonstrates that the optimized MTT protocol yields remarkably higher A570nm (HSS)/A570nm (control) signal than the previously pub-lished MTT assay by approximately twice as many, whereas the tendencies of these two curves are similar, and the corresponding HSS doses of the peak measured by these two methods are almost identical.

3Discussion

One of the most important modification, was concerned with the method to dissolve the crystals of MTT formazan produced. Changing the solvent from the detergent (10%SDS-0.01N HCl) to the pure organic solvent (DMSO) resolved the problems of overnight incubation and incomplete solubilization. DMSO is an attractive solvent for its rapidness, thorough solubilization and high absorbance signal representing the sensitivity. However, when DMSO is used, most of the medium must be removed from the wells prior to the addition of DMSO. There will always be a risk of losing some formazan in the process, and this could contribute to the higher standard deviations on quadruplicate determinations.

The majority of the mitochondrial enzyme succinate-dehydrogenase is distributed over surface of membrane, and the environment of enzyme reaction with WTT is pH 6 .7.Some parameters of the MTT assay, such as the presence of serum, phenol red and unconverted MTT were thought to be important on the absorbance signal, so the supernatant was discarded after the incubation with HSS and before the addition of DMSO.

The number and metabolic of cells could have a crucial influence on the MTT assay[15]. High concentration of cells renders measurement impossible, either be-cause the absorbance value is too high to be within scale of ELISA scanner, or because the

cell grows fast by stimulating of HSS and fills up the well of 96-microwell culture plate in a short time, which results in the less sensitivity of the MTT assay. Furthermore, the metabolic status of cells must be similar in experiments comparing the number of cells since differences in metabolic status may otherwise give rise to different A values from cell cultures containing the same number of cells.

The stimulation of SMMC 7721 cells represents a true increase as measured by the optimized criterion over a wide range of HSS dose from 0 to 20 μg: a significant growth in cell number of HSS-treated culture compared with that of control($P<0.001$). However, it is interesting to note that the curve drops when HSS dose beyond a certain value. Studies on investigating the mechanism will require confirmation with normal hepatocytes to avoid effect of a specific hepatoma cell stimulator that might have no relevance to normal liver growth.

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