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MTT Vs WST-1, efficiency, cost, time, and waste generation: evaluating the silver nanoparticles cytotoxicity

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Palabras clave
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Resumen

Los ensayos de MTT y WST-1 para determinar la viabilidad celular se utilizan con frecuencia, sin considerar una opción diferente; sin embargo, ambos métodos requieren procedimientos y materiales específicos que no son comunes entre ellos y por lo tanto implican una base teórica diferente, los cuales son aspectos importantes a señalar cuando es necesario elegir un ensayo de manera consciente.

Por otro lado, las nanopartículas metálicas, en su mayoría sintetizadas por vía química, se estudian en el área de la salud como alternativa a los agentes terapéuticos; sin embargo, se ha

demostrado que son tóxicos debido a su entorno de protección y subproductos de reacción que son difíciles de purificar. De esta manera, se ha demostrado que los métodos de síntesis más ecológicos producen nanopartículas con menos reactivos y, a menudo, se sugiere o se espera una toxicidad más baja. En este trabajo se realizó una comparación entre los métodos MTT y WST-1 para evaluar la citotoxicidad de las nanopartículas de plata, utilizando nanopartículas cubiertas con extracto de hojas de *Ficus benjamina* (*F. benjamina*) y nanopartículas cubiertas con moléculas de citrato. Se evaluó la citotoxicidad de las nanopartículas obtenidas por ambos métodos, así como parámetros de cultivo celular como costos, tiempo y productos de desechos generados. Los resultados mostraron que la eficiencia para determinar la viabilidad celular en ambos métodos fue similar (p> 0.05): nanopartículas de plata cubiertas con biomoléculas de hojas *de F. benjamina* mostraron una disminución> 60% de las células viables a una concentración de 13 µg mL^{-1}, mientras que las nanopartículas cubiertas con moléculas de citrato solo disminuyeron el 20% aproximadamente a una concentración de 25 µg mL^{-1}. Hablando del costo del análisis, el costo económico del ensayo WST-1 fue 3.4 veces mayor que el del ensayo MTT. Por otro lado, el ensayo MTT requirió 2,5 h más y produjo más de tres veces el volumen de desechos que el ensayo WST-1.

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Abstract

MTT and WST-1 are the cell viability assays frequently used without even considering a different choice; however, both methods require specific procedures and materials that are not common among them and thus implies a different theoretical basis, which are important aspects to denote when is necessary to choose an assay consciously.

On the other hand, metallic nanoparticles, mostly synthesized by a chemical route, are studied in the health area as an alternative for therapeutic agents; however, they have proven to be toxic due to their capping environment and reaction by-products that are difficult to purify. In this way, the greener synthesis methods have shown to produce nanoparticles with fewer reagents, and a lower toxicity is often suggested or expected. In this work, a comparison between MTT and WST-1 methods was performed to evaluate the cytotoxicity of the silver nanoparticles using nanoparticles capped with a *Ficus benjamina* (*F. benjamina*) leaves extract and nanoparticles capped with citrate molecules. The cytotoxicity of the nanoparticles obtained by both methods were evaluated, as well as cell culture parameters such as costs, time, and generated products and wastes. The results showed that the efficiency to determine the cell viability in both

methods were similar (p> 0.05): silver nanoparticles capped with biomolecules from *F. benjamina* leaves showed a >60% decrease of the viable cells at a concentration of 13 µg mL^{-1}, while nanoparticles capped with citrate molecules exhibit a decrease of 20% approximately at a concentration of 25 µg mL^{-1}. Talking about the analysis cost, the WST-1 assay economical cost was 3.4 times higher than the MTT assay. On the other hand, the MTT assay required was 2.5 h slower and produced more than three times of the waste volume than the WST-1 assay.

Keywords: MTT, WST-1, silver nanoparticles, *Ficus benjamina*, citrate molecules.

Introduction

The toxicological assays in cell lines are used as screening tests to evaluate compounds with a pharmacological interest and to determine their dangerous biological effects. [1,2] In comparison with the studies made in animals, this type of assays are ethically less ambiguous, easier to control and reproduce, as well as less expensive.[3]

The two methods most widely used to evaluate the cytotoxicity of a compound are based in the reduction of tetrazolium salts to formazan by dehydrogenases, leading to a colored complex, whose quantity can be measured by spectrophotometric equipment and is proportional to the number of viable cells. The reduction of the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) reagent takes place inside the mitochondria forming formazan crystals [4] on the other hand, in the assay with the 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1) reagent, these molecules are reduced both, inside and outside the mitochondria due to its physicochemical characteristics, which allow its solubility in the growth medium (Roche Germany). These assays allow the determination of the cell viability by metabolic activity, mainly based on enzyme activity evaluation, and this shows that only a viable cell will react with the compound in a way that if the cell is not capable of performing this process, is because the cell is dead, as the enzymatic activity can only be performed by living cells. Although MTT and WST-1 are commonly performed to study cell proliferation assays, there are no reports that remark the significant differences or advantages and disadvantages of both methods.

On the other hand, the metallic nanoparticles (NPs) are used in diverse industrial fields due to their mechanical, chemical, and electrical properties. Nowadays, in the health area, it is considered that some nanomaterials could have potential use in the development of drugs, biological markers for diagnosis, molecular vectors as well

as in the search for potential cancer treatments and infectious diseases taking advantage of their morphology-related properties.[5] However, in multiple studies, the toxicity of metal nanoparticles synthesized by chemical methods has been observed. Green synthesis methods use molecules from plant extracts to reduce and stabilize nanoparticles, which represents an advantage in terms of the use of toxic and expensive compounds, simplifying the process itself. Nevertheless, it is necessary to study the toxicity of this kind of dispersion and if it is possible to obtain an additional effect derived from the type of extract used during the synthesis.[6]
In this work, we compared WST-1 and MTT methods for determining the cytotoxicity of silver nanoparticles. In addition to efficiency, other parameters such as cost, time, and waste generation were also studied, as well as the effect of the capping agent on the dispersion toxicity when comparing nanoparticles synthesized with the conventional chemical method and through green synthesis.

Material and methods

Nanoparticles (NPs)

Two spherical silver nanoparticle dispersions, capped with citrate (AgNP-C) and capped with extract of *F. benjamina* (AgNP-FB) [7,8]

Cell proliferation assay

Cell line

Chang liver cell line was obtained from American Type Culture Collection (ATCC).

Cell line culture and exposure to the NP

Cells were cultured in Minimum Essential Medium (MEM) (Sigma Aldrich), supplemented with 10% fetal bovine serum (FBS, GIBCO) and 1X penicillin/streptomycin (Sigma Aldrich). Cells were incubated under a humidified atmosphere containing 5% CO_2 at 37 °C and subcultured until 80% confluent. Briefly, cells were seeded at a cell density of 10×10^4 per 100 µL medium on each well of a 96-well plate and cultivated for 24 h. Afterwards, the cells were exposed for 24 h to the nanoparticle dispersions at a range of concentrations between 3.125 and 200 µg mL^{-1}. Cells and culture medium were used as negative control; whereas cells and culture medium with 1% triton in PBS was used as the positive control. Subsequently, cell viability is determined by the methods under study. All experiments were conducted in triplicate.

Evaluation of cell viability using MTT and WST-1 assays

After the period of incubation, the medium was removed, and the plates were washed twice with phosphate buffer. To the first plate, 100 µL of MEM containing 0.5 mg/mL MTT (SIGMA, USA) dissolved were added; to the second plate, 100 µL of MEM plus 10 µL of WST-1 reagent (ROCHE, Germany) were added. The plate with MTT was incubated for 3.5 h, while the plate with WST-1 was incubated for 2 h, both plates at 37 ºC in an environment of 5%/95% CO_2/air. After 2 h, the optical density of the plate that contained WST-1 was measured at 450 nm with ELISA lector (BioTec ELX800). On the other hand, after its incubation time, the medium containing MTT was removed from the dish and replaced with 200 µL of acidified isopropyl alcohol in order to dissolve the formazan crystals, and it was kept in dark conditions during 30 min, and finally, it was measured at 570 nm.[4] For the estimation of the viability percentage of the cells exposed to the different nanoparticle concentrations we use the control that only contained cells and medium, which showed 100 percent after 24 h.

Analysis

Efficiency and Viability. The difference in the viability of the methods used was evaluated with a 2-way ANOVA test, followed by the Bonferroni test, for which a $p<0.05$ was considered a significant value. For this, the statistical software NCSS version 11 was used.

Costs. The cost of each reagent and material was verified and used to calculate the cost of running a 96-well plate for each method.

Time. The minimum time required to run a 96-well plate starting from the cell culture and finishing after the plate spectrophotometric lecture with the corresponding indicator were made, was also recorded for both assays.

Waste generation. The compounds used were listed starting from the addition of WST-1 and MTT, as well as the subsequent compounds produced until the lecture of the plate, were also measured. A search of the toxicological properties in renowned databases to analyze and compare the sustainability of the techniques was also carried out.

Results

Evaluation of the NPs cytotoxicity by MTT and WST-1 assays

The percentage of cell viability in chang liver cells exposed to Ag NPs with different capping did not show a significant difference between the methods WST-1 and MTT when citrate-capped ($p = 0.72$) and *F.*

benjamina-capped (p = 0.06) NPs were evaluated. (Figure 1). In the case of the Ag NPs capped with citrate molecules, called nanoparticles by chemical or inorganic synthesis, similar behaviors were observed.

Nonetheless, in the case of MTT, the cell viability decreased 80% at a concentration of 3 µg mL^{-1}, behavior that kept up until around 36% approximately at the concentration of 200 mg mL^{-1}. In the case of the assay with WST-1 a similar behavior was observed, but the viability values are around 10% greater than MTT for each concentration (Figure 1a).

For the case of AgNP-FB or green synthesized nanoparticles, the cell viability decreased until an 80% with a concentration of 6 µg mL^{-1}, however, at the concentration of 13 µg mL^{-1}, it had a drastic decrease until the 50 and 60% of the viability for WST-1 and MTT. In the final concentrations, cell viability decreased to 37 – 50% (Figure 1b).

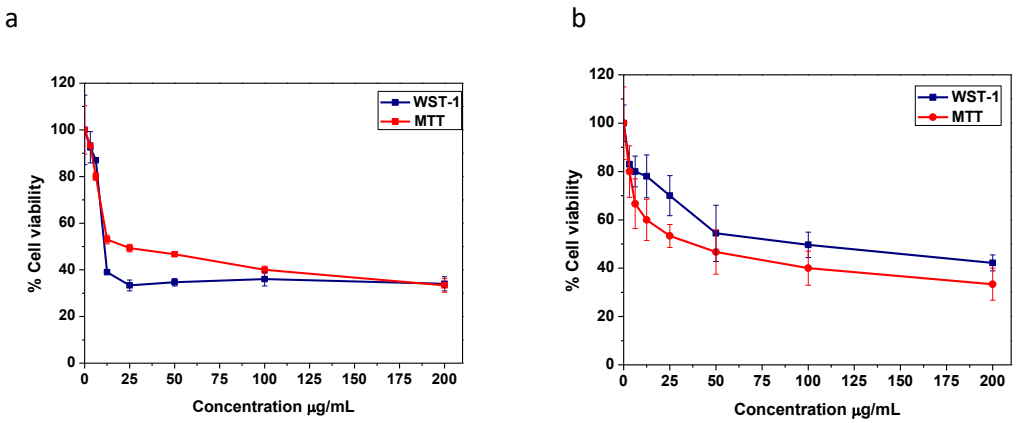

Figure 1. Comparison of the percentage of cell viability of silver nanoparticles stabilized with the extract of *F. benjamina* (a) and capped with citrate molecules (b) by the assays with WST-1 and MTT at 24 h of exposure. By triplicated in 3 different days.

Costs analysis

The results obtained in this study, show that the cost of the WST-1 assay is around 14 dollars per 96-well plate, this cost is three times more expensive than the assay with MTT, which is around 4 dollars.

Time analysis

The analysis of the used time to perform each assay showed that 4 h are spent in the MTT assay and 2 h in the WST-1 assay.

Waste analysis

Regarding waste generation, 9.6 mL of waste is generated in the WST-1 assay and 28.8 mL with the MTT assay: 8.64mL of medium plus 0.96 mL of WST1; and 4.8 mg of MTT, 9.6 mL of medium, 19.2 mL of isopropyl alcohol, plus 1mL HCl.

Discussion

In this work, we compared two of the most used assays to evaluate the cellular proliferation, MTT, and WST-1. There are multiple reports where these methods are used to evaluate the cytotoxicity of compounds already known like lidocaine and desflurane, [9,10] compounds in investigation with potential anticancer activity, [11,12] prostaglandins analogs,[13] compounds with potential antimicrobial effect,[14] immunosuppressants,[15] and even nanomaterials, which have been a discussion topic in different areas, especially in the heath area, like the silver nanoparticles case. [16]

Although we do not find a significant difference between the two methods, it was observed a different pattern in the cytotoxicity depending on the capping agent of the nanoparticle. The AgNP-FB showed low cytotoxicity in the first concentrations, but at the concentration of 13 µg/mL the cellular viability which was constant until the last concentration evaluated diminishes considerably. With respect to AgNP-C, the viability decreases with regard to concentration in a gradual way. This different pattern between AgNP-FB and AgNP-C could be due to greater stability of the later nanoparticle, their size is more uniform and theoretically the liberation of silver ions is more stable.[16] More experiments are needed, to confirm it.

Some authors compared both methods for the evaluation of the same compounds concluding that the MTT is more sensible or stable than the WST-1, but the latter is faster,[17,18] without taking into account other comparable parameters. Even other authors mention the evaluation of the cytotoxicity with MTT and the oxygen reactive species with the inhibition of the reduction of WST-1,[19] demonstrating that this reagent can have diverse applications,[20,12] evaluate compounds of selenium and compared the effectiveness of the use of various types of assays and observed that the assays where it was used WST-1 and MTT were not sensible at low concentrations used

and vital dyes like neutral red and brilliant blue shown to be more effective and comparable; also they discuss that the effectiveness of the method is related to the type of chemical that is being.[12] The authors who made the comparison of these two techniques only paid attention to the sensibility, effectiveness, or quickness; most of them agree that the MTT is more sensible, even though the WST-1 is faster. In our case, further the evaluation of these two variables, the cost and the generation of residues was also evaluated.[17, 18, 19, 22] Therefore, regarding the toxicity, both indicators of mitochondrial activity belong to the same group of salts and no significant difference could be found. However, the MTT method uses acidified isopropyl alcohol and its toxicological properties are dermal and respiratory irritation and damage to the nervous system by overdose and rarely cause death; however, at the concentrations used and in the way it is handled, it is not considered dangerous for the user or for the environment since suitable containers are used in the research laboratory, also, it is not an expensive reagent either. This makes us conclude that the method with WST-1, despite not using additional reagents, is the most expensive. [23]

Conclusions

In conclusion, the estimation of cell viability is similar in both methods. The MTT assay is more stable and less expensive; however, the WST-1 has a low process-time, simplicity, and generate fewer residues or waste.

It is important to choose a suitable method for each molecule, compound, or nanoparticle to be evaluated. In addition to the parameters studied, the solubility and stability of the product must be considered.

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