JBUON 2018; 23(5): 1505-1513
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

A simplified colorimetric method for rapid detection of cell viability and toxicity in adherent cell culture systems

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Summary

Purpose: *Evaluation of cell viability and toxicity in adherent culture systems is of critical relevance for a wide range of disciplines of biomedical sciences research, including cancer research, toxicology, pharmacology, cell biology, neurology and nanomedicine. Several well-established cytotoxicity assays are widely used by researchers, including the most-preferred MTT assay. Nevertheless, there are problems associated with them, for example; in terms of the time-factor and solubilization of the formzan crystals before its spectroscopic quantification. In this study, we propose a simple, fast and cost-effective colorimetric assay that is free of these issues.*

Methods: *Our assay was based upon reductive splitting of blue-green colored supravital safranin derivative dye Janus green B (JG-B) to pink colored diethyl safranin by oxidoreductases of the electron transport chain (ETC) of actively respiring mitochondria. Because this conversion can be easily and reliably followed spectroscopically, measure of diethyl safranin formed from extraneously added JG-B provides a proficient indicator of cellular health and viability.*

Results: *Using MCF-7, a breast cancer cell line, we provide a proof of concept for the suggested assay and compare it with the MTT assay.*

Conclusions: *Unlike the MTT assay, our JG-B assay does not require a solubilization/extraction step, and hence follows a much simpler and time-efficient protocol suitable for high-throughput analysis of cell viability in anchorage-dependent cell culture models. Additionally, the JG-B cell viability assay reported here can be suitably applied either independently or in complementation with other assays for the analysis of cellular viability and toxicity in both analytic and therapeutic aspects of research.*

Key words: *cancer cells, cisplatin, cytotoxicity, Janus green B, mitochondria, MTT*

Introduction

The analysis of cell viability in adherent culture systems is routinely performed in cell culture laboratories because of its indispensability in the assessment of cell viability and toxicity in response to extracellular activators, toxic stressors and potential pharmaceuticals. Several protocols are available and some are currently being used for the assessment of cell viability. Colorimeter is the method of choice for rapid and economical assessment of cell viability, and several dyes and protocols are currently used for this purpose. These include neutral red that binds to lysosomes

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Received: 02/02/2018; Accepted; 01/03/2018

[1], propidium iodide that binds to the DNA of the damaged cells [2], crystal violet that binds to the proteins and DNA [3], Alamar blue (resazurin; Al-Nasiry et al. [4]) and Janus green B that binds to mitochondria [5]. Nevertheless, tetrazolium salts like 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) are currently the most widely employed agents for colorimetric assay of cell viability [6,7].

However, several types of agents have been shown to reduce MTT leading to erroneous results in cell viability assays. These include serum albumin [8], metal ions [9,10], plant extracts and antioxidants [11,12], vitamins [13] pH of medium [14,15] and concentration of glucose, NADH and NADPH [16]. Nevertheless, many of these constraints can be dealt by extensively washing the cultured cells before the initiation of exposure to MTT [17]. Hence, there is a critical requirement for the employment of complementary cell viability assay for the validation of the results and inferences obtained from the well-established cell viability assay.

In addition to the above stated limitations, the MTT assay also requires solubilization/elution of formazan (the water-insoluble reduction product) before the spectroscopic analysis [17]. This makes MTT assay tedious and time-consuming and, in addition, potentially error prone [18]. In this regard, tetrazolium salts like 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), which are designed to produce water-soluble formazan are suitable alternatives. However, the employment of XTT in cell viability assessment has been limited because of the net negative charge of XTT accountable for its largely cell impermeable nature [6].

To bypass the time-consuming solubilization/extraction step central to MTT cell viability assay, we have designed a cheap, time-efficient and high-throughput assay for the quantitative analysis of cell viability and toxicity. Briefly, the assay protocol employs 8-[[4-(dimethylamino) phenyl]diazenyl]-N,N-diethyl-10-phenylphenazin-10-ium-2-amine chloride, commonly referred to as Janus green B (JG-B). JG-B is a supravital lipophilic cationic mono-azo safranin derivative and like other lipophilic cationic dyes including MTT, JG-B is taken up only by active mitochondria with an intact negative inside membrane potential [19]. Unlike MTT which is reduced by mitochondrial dehydrogenases from a yellow colored dye to water-insoluble blue formazan product, JG-B is reductively split from a deep greenish-blue color dye (absorption maximum at 595 nm) to pink color water-soluble diethyl safranin (absorption maximum at 550 nm) [20-23]. Hence, the formation of diethyl safranin can be directly measured spectrophotometrically without any requirement of its extraction or elution.

The uptake and accumulation of JG-B by actively respiring mitochondria with a negative membrane potential in adherent cells has been employed previously to analyze the cell numbers by some research groups [5,24-27], although the assay is time-consuming and requires error prone steps of ethanol-fixation, washing and de-staining preceding spectroscopic analysis of JG-B at 595 nm [5,26]. Moreover, the analysis of cellular uptake of JG-B as reported previously [5,26] could lead to erroneous results because JG-B can non-specifically bind to nuclei and dead cells as well as non-mitochondrial cellular compartments [28]. On the other hand, the JG-B cell viability assay proposed in this study employs the principle of reductive splitting of JG-B into diethyl safranin and involves the measurement of the absorbance ratio at 550 nm/595 nm, circumventing both the de-staining (previously used JG-B assays) [5,26] and solubilization steps (MTT assay) [17,18]. This is a major advantage when considering the robustness, simplicity and time consumption factors.

Employing the principle of reductive splitting of JG-B into diethyl safranin by actively respiring mitochondria in view, we propose a simple and rapid colorimetric assay for the assessment of the viability of the cells in anchorage-dependent culture systems. In this study, we provide a proof of principle of our JG-B cell viability assay and its comparison with MTT assay, providing evidence for its suitability as a time-efficient, high throughput and robust assay for the analysis of cell viability/integrity and toxicity, both independently and in complementation of the established cell viability protocols based on MTT and neutral red.

Methods

Chemicals and reagents

All the chemicals/biochemicals used in this study were of analytical grade and procured from Merck (Darmstadt, Germany) or Sigma-Aldrich (Steinheim, Germany). Janus green B and MTT were procured from Merck and Sigma, respectively.

Mitochondrial reduction of JG-B

In order to assess the reduction of JG-B by the isolated mitochondria, 10 µM JG-B with varying amounts of highly enriched mitochondrial preparations or post-mitochondrial supernatant (PMS) were obtained from rat brain cortices (Supplementary methods). Absorbance was recorded at 550 and 595 nm before and 10 min after

the addition of mitochondria or PMS and the formation of diethyl safranin was measured as a ratio of absorbance at 550 nm/595 nm. All the experiments involving animals were carried out in accordance with the institutional guidelines for animal care and use in scientific research and after approval from the Institutional Review Board (IRB), Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia.

Cell culture

MCF-7 breast cancer cells were maintained at a subconfluent state in DMEM medium containing 10% fetal calf serum (FCS), 200 µg/ml streptomycin, 200 U/ml penicillin in humidified conditions (95/5% air/CO_2) at 37°C. A well-known anti-cancer drug, cisplatin, was used for inducing cell death in MCF-7 cells. For cisplatin treatment, MCF-7 cells were seeded at 25,000 cells per well of 96-well culture plates (Thermo Fisher Scientific, USA) to reach a 50-60% confluence. Varying concentrations of cisplatin were added 24 hrs after seeding and the cells were further cultured for another 48 hrs before processing for MTT or JG-B assay.

MTT cell viability assay

MTT assay is based on the principle of the formation of deep purple colored formazan upon the reduction of MTT by dehydrogenases of actively respiring mitochondria (Supplementary Figure 1A). MTT cell viability assay was carried out as reported earlier [7,29] with slight modification. Briefly, the cells in 96-well plate format were washed twice with phosphate buffered saline (PBS) pH 7.4 and incubated with 0.5 mg/ml of MTT at 37°C for 4 hrs. Subsequently, the excess of unbound MTT was aspirated and formazan crystals formed were solubilized in 150 µl of isopropanol-based solubilizing solution containing 4 mM HCl and 0.1% Nondet P-40 (NP-40) dissolved in isopropanol. The solubilized formazan crystals were incubated in the dark at room temperature for 15 min with agitation before being measured spectrophotometrically at 570 nm.

JG-B cell viability assay

Likewise MTT, JG-B undergoes reductive splitting upon the action of electron transport chain (ETC) oxidoreductases of the actively respiring mitochondria to form diethyl safranin (Supplementary Figure 1B). For the assessment of cell viability using JG-B, cells were washed twice with PBS pH 7.4 and incubated with 10 µM JG-B at 37°C in the dark for 60 min. The absorbance at 550 and 595 nm was recorded immediately after the addition of JG-B (i.e. t=0 min) and 60 min (t=60 min) after JG-B incubation. The ratio of absorbance at 550 and 595 nm has been previously employed as a robust measure of diethyl safranin formation from JG-B [30]. Hence, we depicted cell viability as a measure of diethyl safranin formation by measuring the difference of the absorbance ratio 550/595 nm at t=60 min from that at t=0 min. All the spectrophotometric measurements were carried out in 96-well plates using Infinite M200 PRO plate reader (Tecan, Grodig, Austria).

Statistics

Statistical comparisons between the multiple groups was done by one-way analysis of variance (ANOVA) followed by post-hoc tests with Dunnett's correction. Linear or least square non-linear regression analysis of the data was performed using GraphPad Prism 5 software program (GraphPad Software, Inc.). The graphical representation of data was also done using GraphPad Prism 5 software.

Results

Absorption spectra of JG-B and its derivative diethyl safranin

Absorption spectra of blue-green colored JG-B and its pink colored reductive cleavage product diethyl safranin obtained after the treatment of sodium dithionite [30] were recorded and showed absorption maxima at 595 nm and 550 nm for JG-B and for diethyl safranin, respectively (Figure 1A, Supplementary Figure 1).

Mitochondrial reductive splitting of JG-B to diethyl safranin

The enrichment/purity of mitochondria prepared from rat cerebral cortices was confirmed by analyzing the relative levels of SDH activity assay (Supplementary Methods). Around 8-fold increase in the activity of SDH in the purified mitochondrial fraction was observed as compared to the homogenate (Supplementary Figure 2), which indicated a robust enrichment of mitochondria. In addition, the integrity of mitochondrial inner membrane was confirmed by employing the fumarase assay (Supplementary Methods) and it was found to be 93.57 ± 3.20 % (mean ± SD).

In order to confirm the reduction of JG-B to diethyl safranin specifically via mitochondria, the absorption spectrum of JG-B incubated with mitochondria was recorded and compared with free aqueous JG-B. As shown in Figure 1B, an absorption peak was observed at 550 nm (corresponding to diethyl safranin) for JG-B, only when incubated with mitochondria (but not mitochondria-devoid PMS), confirming that mitochondria specifically reduce JG-B to diethyl safranin. Further, the incubation of JG-B with increasing amounts of mitochondria resulted in a dose-dependent enhancement in reductive splitting of JG-B to diethyl safranin. A significant linear increase in the ratio of absorbance at 550 nm /595 nm was observed, indicating reduction of JG-B to diethyl safranin followed a linear trend with increase in mitochondrial dehydrogenases (Figure 1C). Interestingly, the incubation of JG-B with increasing amounts of PMS which was

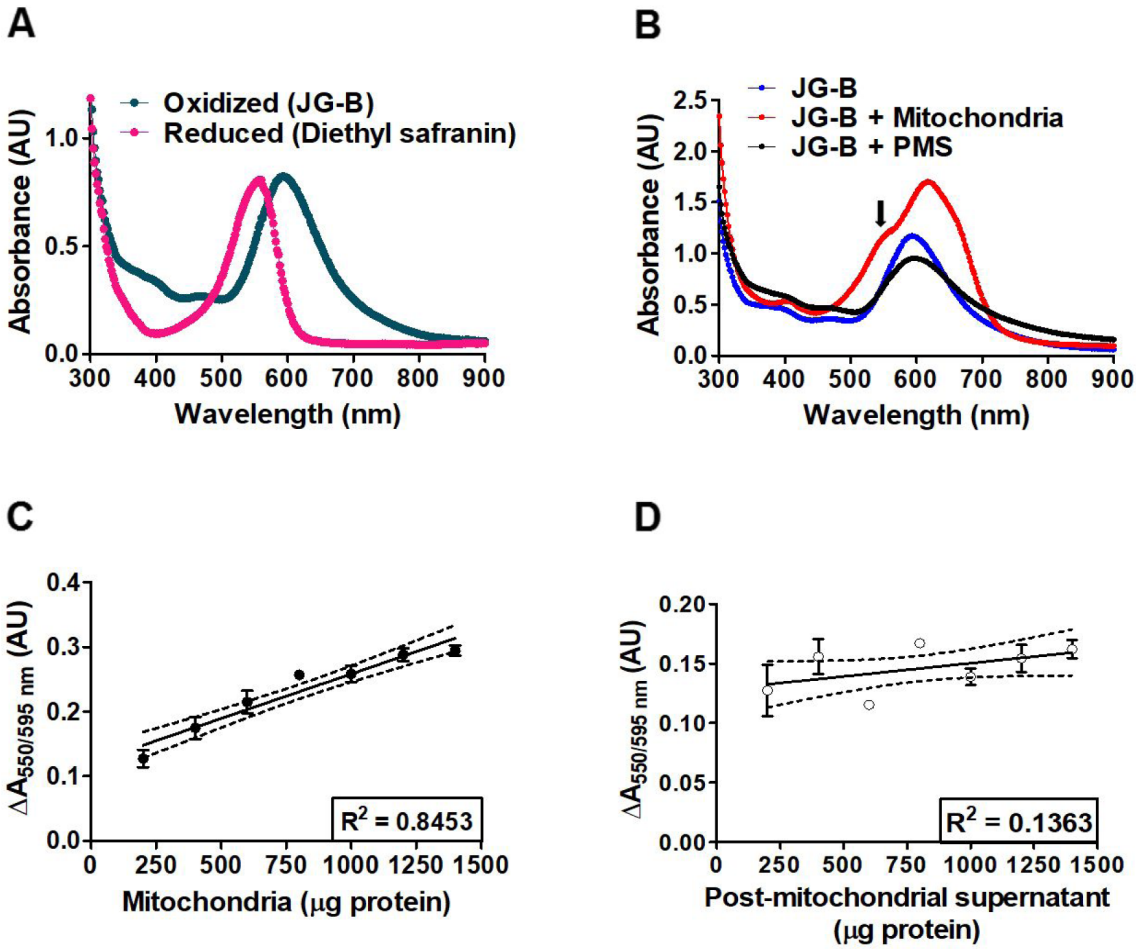


Figure 1. Reductive conversion of JG-B to diethyl safranin is specific for mitochondria and follows a linear trend with increase in mitochondrial amount. **(A):** Absorption spectra of JG-B and diethyl safranin (obtained after sodium dithionite mediated reductive splitting of JG-B) are shown. **(B):** Absorption spectra of JG-B alone or incubated with mitochondria or post-mitochondrial supernatant (PMS) which is largely devoid of any mitochondria are shown. Conversion of JG-B to diethyl safranin occurs only in the presence of mitochondria, as observed as a peak at 550 nm (marked with an arrow). **(C):** Reductive cleavage of JG-B to diethyl safranin, measured as absorbance ratio (550 nm/ 595 nm) increases with increase in mitochondrial amounts and follows a linear trend. **(D):** Exposure of JG-B to PMS does not lead to formation of diethyl safranin. Data is represented as mean ± SEM (n=3;p<0.0001 for **C** and p 0.0995 for **D**).

essentially devoid of all mitochondria, did not result in increase in the absorbance ratio (550 nm /595 nm; Figure 1D), indicating that mitochondria are responsible for most of the conversion of JG-B to diethyl safranin.

JG-B to diethyl safranin conversion follows a linear trend with increasing cell densities

As a proof of concept for the JG-B cell viability assay, we compared it with the well-established MTT assay. For this, MCF-7 cells were seeded in increasing number in 96-well format and correlation graph was established (Figure 2A) for the reduction of JG-B to diethyl safranin (measured as the absorbance ratio at 550 nm/ 595 nm). For the comparison, we also analyzed the cell numbers using MTT assay (Figure 2B) and observed comparable linear curves for both JG-B and MTT protocols.

Cisplatin mediated cytotoxicity in MCF-7 cells is detected efficiently by JG-B

In order to ascertain the effectiveness of the developed JG-B assay for the detection of compromises in cell viability upon exposure to cytotoxic agents, we chose cisplatin (a well-known platinum-based cytotoxic anti-cancer agent) that has detrimental effect on cancer cell lines including MCF-7 [31-33]. Upon exposure of MCF-7 with varying concentrations of cisplatin, we observed a dose-dependent reduction in the cell viability as assessed by JG-B assay (Figure 3A). In addition, MTT assay depicted a comparable loss of cell vitality with increasing concentration of cisplatin (Figure 3B). Furthermore, we observed a strong and significant non-linear correlation between the increasing cisplatin concentration and increasing cytotoxicity as evaluated by JG-B and MTT assays

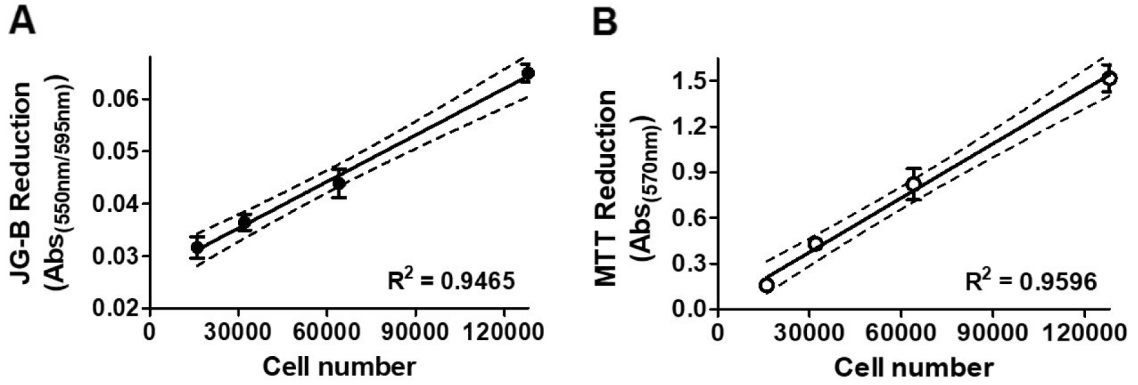


Figure 2. Spectroscopic analysis of formation of diethyl safranin by reductive splitting of JG-B forms an efficient mode for assessing cell numbers. **(A):** Exposure of JG-B to MCF-7 cells leads to its reduction to diethyl safranin that is measured as increase in absorbance ratio (550 nm / 595 nm). Increase in diethyl safranin formation follows a linear trend with increasing number of cells. **(B):** Formation of formazan from MTT in presence of MCF-7 cells as assessed by absorbance at 570 nm, also followed a similar linear trend with increase in cell number per well. Data is represented as mean ± SEM (n=9; 3 triplicates;p<0.0001 for both **B** and **C**).

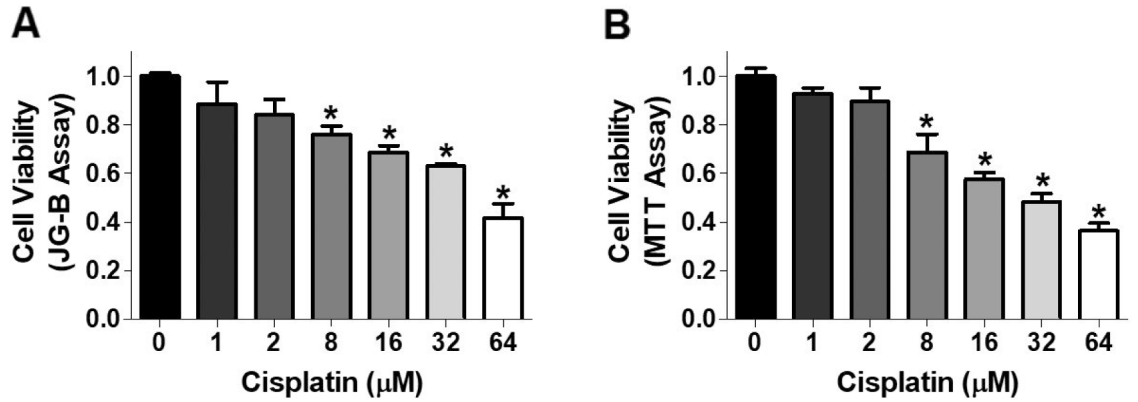


Figure 3. Colorimetric analysis of diethyl safranin formation from JG-B can be effectively employed to measure cell viability. **(A):** MCF-7 cells were treated with varying concentrations of cisplatin and then exposed to JG-B. Formation of diethyl safranin was increasing retarded in cells exposed to increasing concentrations of cisplatin. **(B):** MTT assay also revealed a similar reduction in cell viability upon exposure of MCF-7 cells to increasing concentrations of cisplatin. Respective values are depicted as a ratio of the control value (0 μM cisplatin) and data is represented as mean ± SEM (n=9; 3 triplicates) and * represents statistical significance (p<0.05; ANOVA followed by post-hoc tests with Dunnett's correction).

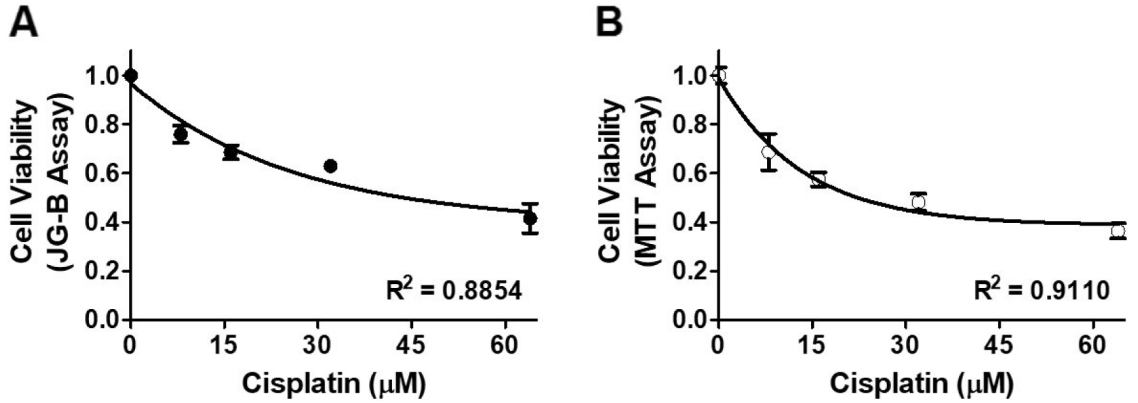


Figure 4. Cisplatin induced cell death follows non-linear relation with increase in its concentration as assessed by JG-B and MTT assays. Both JG-B **(A)** and MTT **(B)** assays reveal comparable statistically significant non-linear negative trend between concentration of cisplatin and number of viable MCF-7 cells. The curves were fitting after least square nonlinear regression analysis. Respective values are depicted as a ratio of the control value (0 μM cisplatin) and data is represented as mean ± SEM (n=9; 3 triplicates).

(Figure 4). The results indicated that the colorimetric JG-B assay is suitable for a robust, consistent and easy quantitative analysis of both cell numbers and cell viability in adherent cell culture systems.

Discussion

JG-B is well known to biomedical science researchers as it has been extensively used for the staining of mitochondria and assessing its purity, integrity and metabolic activity using microscopy-based methods [34-43]. Of note, JG-B has also been employed in the past to assess the cell numbers by some research groups [5, 24-27]. However, all these studies have evaluated the cellular uptake of JG-B utilizing the property of JG-B to accumulate in actively respiring mitochondria with a negative membrane potential, and, as such, the assay requires ethanol-fixation, washing and de-staining steps preceding spectroscopic analysis of JG-B at 595 nm [5,26]. In contrast, the JG-B cell viability assay proposed in this study utilizes the property of reductive splitting of JG-B to diethyl safranin and involves measurement of the absorbance ratio at 550 nm/595 nm, circumventing both the de-staining (previously used JG-B assays) [5,26] and the solubilization steps (MTT assay) [17,18]. This is a major advantage when considering both the simplicity and the time consumption factors. In addition, measuring cellular uptake of JG-B at 595-610 nm as done previously [5,26] could lead to erroneous results because JG-B can non-specifically bind to nuclei of dead cells as well as non-mitochondrial cellular compartments [28].

On the other hand, as diethyl safranin is specifically formed by mitochondrial reduction of JG-B (Figure 1) [21,22,28], measuring its formation would constitute a better measure of mitochondrial activity and hence, cell viability. We have used the ratio of absorbance at 550 and 595 nm to measure the formation of diethyl safranin from JG-B, because the relative amounts of diethyl safranin and JG-B can be calculated using the ratio of absorbance at 550 nm (absorption maximum of diethyl safranin) and 595 nm (absorption maximum of JG-B) [30].

Overall the suggested JG-B cell viability assay provides an effective, simple, economical and high throughput method for the assessment of cell viability and toxicity both as an independent as well as complementary assays for the cell viability in complementation with the established assays which employ neutral red and MTT. As a limitation, there is a critical factor that should be discussed while using JG-B for evaluating the cell viability and toxicity; it should be noted that mitochondrial tracking experiments have shown that over extended periods of time, diethyl safranin is itself converted to leucosafranin by mitochondria, resulting in decolorization of diethyl safranin [21,28,44]. However, this reaction proceeds slowly and decolorization is observed after hours of exposure of JG-B to mitochondrial dehydrogenases, hence it is not a limiting factor for the developed colorimetric assay which requires exposure of the cells to JG-B for 60 min.

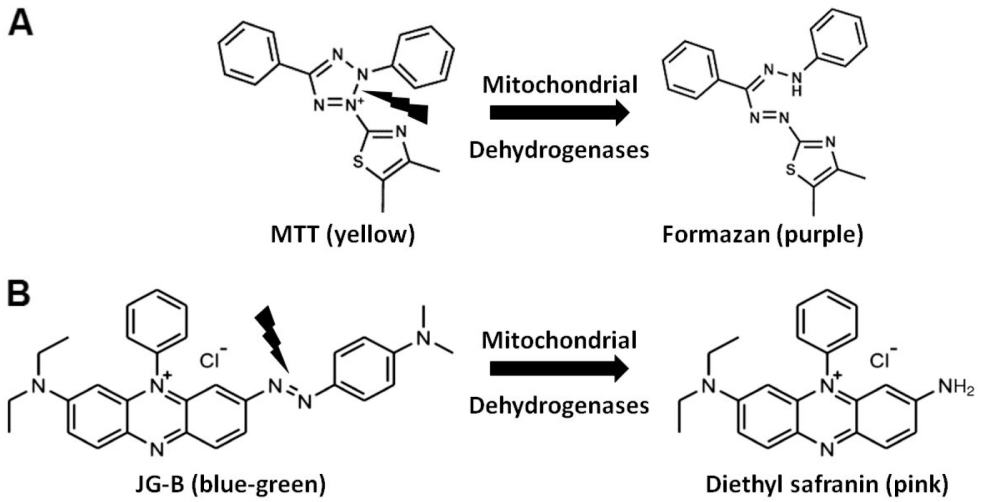
In conclusion, formation of diethyl safranin from JG-B by the action of mitochondrial dehydrogenases (and spectrophotometrically assessed by increase in the ratio of absorbance at 550 nm to 595 nm) can be suitably employed as a basis for a cheap, rapid and consistent assay for assaying the cell viability and toxicity in adherent cell culture models and serve in both analytical and therapeutic research in widely dispersed areas of biomedical research.

Supplementary data

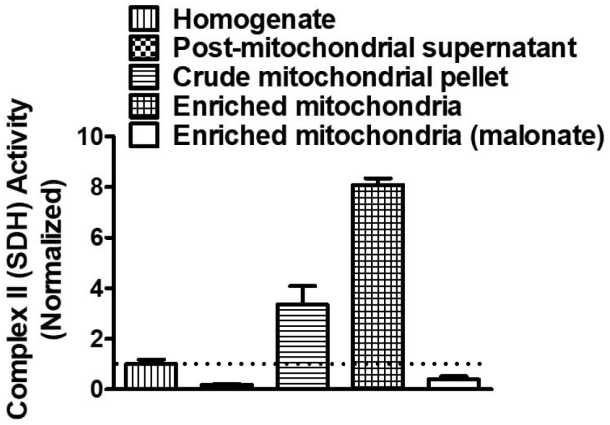
Supplementary methods

Animals: All animal experiments were carried out in accordance with the institutional guidelines for animal care and use in scientific research and after approval from the Institutional Review Board (IRB). Adult Wistar rats were housed in cages under a LD 12/12 regime in rooms with controlled temperature of 25°C with free access to food and water.

Mitochondrial isolation: After decapitation under anesthetic conditions, rat brain cortices were dissected out and mitochondria were isolated using discontinuous sucrose gradient based ultracentrifugation protocol as described previously with slight modifications (Hamberger et al. 1970). In brief, cerebral cortices were homogenized in 10 times volume of isolation buffer (10 mM Tris, 1 mM EDTA(K), 320 mM sucrose, pH 7.4) on ice using a Potter Elvehjem pestle and glass tube. Homogenate obtained was centrifuged at 1300 g for 10 min at 4°C and the supernatant (post-nuclear supernatant) was further centrifuged at 17,000 g for 10 min at 4°C to obtain crude mitochondrial pellet (CMP) and post-mitochondrial supernatant (PMS) which is devoid of any mitochondria. CMP was re-suspended in isolation buffer and loaded onto a discontinuous 1.2-1.0-0.8 M sucrose gradient and centrifuged at 100,000 g for 60 min at 4°C in a Sorvall MX150 micro-ultracentrifuge (Thermo Scientific, Japan). Upon culmination of this step, CMP was separated into three layers; 1) myelin (at first interface of the sucrose gradient), synaptosomes (at second interface) and mitochondria as a pellet. The enriched mitochondrial pellet obtained was washed twice with isolation buffer at 10,000 g for 10 min at 4°C, re-suspended in isolation buffer and aliquoted and stored at -80°C. Purity and integrity of mitochondrial fraction was assessed by enrichment of activity of succinate dehydrogenase (SDH), which is a well-known mitochon-



Supplementary Figure 1. Chemistry of MTT and JG-B reduction. Chemical structures of JG-B and its reduced product diethyl safranin **(A)** and MTT and its reduced product formazan **(B)** are depicted. Thunderbolt sign represents the respective site of reduction in the parent oxidized species.



Supplementary Figure 2. Isolation of purified mitochondria fraction from rat brain cortices by discontinuous sucrose gradient based ultracentrifugation. Enrichment of mitochondrial marker complex II (succinate dehydrogenase) activity was observed for cortical mitochondria isolated using discontinuous sucrose gradient based ultracentrifugation method. SDH activity levels were enriched by 8.1 ± 0.4 (mean ± SD) times compared to homogenate. Data is represented as mean ± SEM (n=3).

drial marker protein; and activity assay of fumarase in presence or absence of detergent Triton X-100, respectively (see below).

Succinate dehydrogenase (SDH) activity assay: To verify mitochondrial enrichment at each stage of purification, SDH activity was measured in homogenate, post-nuclear supernatant (PNS), post mitochondrial supernatant and crude and enriched mitochondrial fractions using a spectroscopic assay described previously [2]. Briefly, the different subcellular fractions (0.1 mg protein each) were suspended in 50 mM (K) phosphate buffer, pH 7.4 containing 3 mM potassium ferricyanide (III) acting as an exogenous electron acceptor. Formation of potassium ferrocyanide (II) was assessed as decrease in absorption at 420 nm upon addition of substrate (50 mM succinate) at 30°C for 2 min. Reaction rate was calculated as nmol ferricyanide reduced per min per mg protein and is represented with respect to SDH activity in the homogenate fraction (ε_{420} for potassium ferricyanide = 1020 M^{-1} cm^{-1}). Purified mitochondria pretreated with 500 mM malonate, a competitive inhibitor of SDH, served as a negative control.

Mitochondrial integrity assay: Integrity of enriched brain cortical mitochondrial fraction was measured using activity assay of fumarase enzyme (marker for mitochondrial matrix) in presence or absence of detergent Triton X-100 [3]. The assay is based upon fumarase mediated formation of fumarate from substrate L-malate which is observed as an increase in absorbance at 240 nm. Increase in absorbance (240 nm) upon addition of 50 mM malate to mitochondria (0.1 mg protein) in the presence or absence of 0.2 % Triton X-100 detergent was measured. The linear part of the kinetic curve was used to calculate the slope of fumarase activity and expressed as nmol fumarate formed/min/mg protein (ε_{240} for fumarate = 2.44 mM^{-1} cm^{-1}). Integrity of brain mitochondria was calculated as $1-(v_{-TX}/v_{+TX})$ where v_{-TX} and v_{+TX} represent fumarase reaction rate in absence and presence of Triton X-100, respectively and expressed as a percentage.

Acknowledgements

This study was partly funded by the Deanship of Scientific Research, Imam Abdulrahman Bin Faisal University (Project No. 2016-087-IRMC). We extend special thanks to Prof. Amin Al-Ali, Department of Biochemistry, College of Medicine, Imam Abdulrahman Bin Faisal University for his helpful scientific suggestions.

Conflict of interests

The authors declare no conflict of interests.

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