An improved 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) proliferation assay to overcome the interference of hydralazine

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ABSTRACT

The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay is one of the most commonly used assays to assess cell proliferation and cytotoxicity, but is subject to interference by testing compounds. Hydralazine, an antihypertensive drug, is commonly investigated in multiple fields such as heart failure, cancer and blood pressure research. This study reported interference of the MTS assay by hydralazine and a simple modification overcoming this interference. Vascular smooth muscle cells were cultured in the presence or absence of hydralazine (0, 10, 50,100 and 500 μM) for 2 h or 24h. Cell numbers were analyzed using MTS, trypan blue exclusion or microscopic assays. A modified version of the standard MTS assay was established in which an additional step was added replacing the test medium, containing hydralazine, with fresh culture medium immediately before the addition of the MTS reagent. Culture with hydralazine at concentrations of 50, 100 and 500 μM for 2h increased absorbance ($P<0.05$) in the standard MTS assay, whereas microscopy suggested no change in cell numbers. Culture with 500 μm hydralazine for 24 h increased absorbance ($P<0.05$) in the standard MTS assay, however trypan blue exclusion and microscopy suggested a decrease in cell numbers. In a cell-free system, hydralazine (≥10 μM) increased absorbance in a concentration-dependent manner. The modified MTS assay produced results consistent with trypan blue exclusion and microscopy. In conclusion, a simple modification of the standard MTS assay overcame the interference of hydralazine and may be useful to avoid interference from other tested compounds.

INTRODUCTION

In viable cells, NAD(P)H-dependent oxidoreductase enzymes expressed in mitochondria are capable of converting tetrazolium into colored formazan.[1] The amount of the formazan is proportional to the metabolic activity of mitochondrial enzymes which are produced by live cells. The amount of produced formazan can be quantified through absorbance measured by a spectrophotometer and used to estimate the cell numbers. Different types of tetrazolium can be used for this purpose.[1-6] The MTS assay uses 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.[1] MTS can be converted to formazan products that are directly soluble in cell culture medium,[1] which is in contrast with the MTT [3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] assay in which the formazan products are insoluble and a proper method to solubilize the formazan product is needed.[1, 7] The MTS assay is therefore often described as a "one-step" MTT assay. The standard MTS assay protocol is very simple, since the MTS reagent is directly added to the cultured cells and then the absorbance measured after a defined period of incubation.

Accurately measuring cell proliferation and cytotoxicity is important in many research fields and the MTS assay is one of the most commonly used assays for this purpose. Hydralazine is an antihypertensive drug and commonly investigated in multiple fields such as heart failure,[8] cancer[9, 10] and blood pressure research[11]. The effect of hydralazine on the MTS assay has not been previously reported. The aim of this study was to examine the effect of hydralazine on cell proliferation and cytotoxicity and the MTS assay. A modified version of the standard MTS assay was established, and tested, in which an additional step was added replacing the test medium, containing hydralazine, with fresh culture medium immediately before the addition of the MTS reagent.

MATERIALS AND METHODS

Cell culture

The primary vascular smooth muscle cells (VSMCs) were isolated from mouse aorta[12] and cultured as previously described.[13] In brief, cells were cultured in Dulbecco's modified Eagle medium (Life Technologies Australia Pty Ltd, Melbourne, VIC, Australia) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Sydney, NSW, Australia), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, Sydney, NSW, Australia) at humidified atmosphere at 37°C in an incubator containing 5% CO_2. The cells were split with 0.05% trypsin/0.02% ethylenediaminetetraacetic acid when they reached 80% confluency and sub-cultured for further passages. The use of animal tissues was approved according to the principles consistent with the 2013, 8th Edition "Australian Code for the Care and Use of Animals for Scientific Purposes" (National Health and Medical Research Council of Australia).

MTS assay

The standard MTS assay: Cells were seeded at a concentration of $1x10^5$ cells /ml, 200 μl/well into 96-well flat bottomed tissue culture plates in 8 replicates. The MTS assay was carried out using the MTS Cell Proliferation Colorimetric Assay Kit (BioVision Inc, Milpitas, CA, US) following the manufacturer's instruction. In brief, after the cells were cultured with hydralazine (0, 10, 50, 100 and 500 μM) or PBS for 2 h or 24 h, 20 μl of MTS reagent was added into each well and the cells were further incubated for 2-4 h at 37°C in standard culture conditions. Then the absorbance was detected at 490 nm with a Microplate Reader.[14]

The modified MTS assay: This was the same as the above-described method except that the culture medium in the well was aspirated and replaced with 200 μl fresh, pre-warmed standard cell medium immediately before the addition of the 20 μl of MTS reagent. The aspiration was conducted gently with a glass pipette which was connected to an electric vacuum pump. The

glass pipette touched only a small spot on the bottom of a well at the end of the aspiration procedure. The liquid in the well was completely aspirated and cell loss beyond the small spot where the glass pipette touched was minimal.

Trypan blue exclusion assay

2 mL of VSMCs ($1x10^5$/ml) were placed in a flat-bottom 6-well plate for 24 h. 4 µL of PBS or various concentrations of hydralazine were added to give a final concentration of 0, 10, 50, 100 and 500 µM hydralazine. 24 h later, the cells were trypsinized. 10 µL of cell suspension was mixed with 10 µL trypan blue and cell numbers were counted using an automated Cell Countess cell counter (Thermo Fisher Scientific, Melbourne, VIC, Australia).[14]

Imaging using light microscopy

Images of the cell culture plates were captured using a contrast phase microscope (Thermo Fisher Scientific, Melbourne, VIC, Australia).

Statistical Analysis

SPSS (Version 25) was used for all statistical analyses. Data were presented as Mean ± standard deviation. Comparison of means was performed by one-way analysis of variance with Bonferroni post hoc test. Differences were considered to be statistically significant at $P<0.05$.

RESULTS

Over-estimate of live cell numbers by the standard MTS assay

In the cells incubated with hydralazine for 2 h, the results obtained from the standard MTS assay showed that hydralazine dose-dependently increased the absorbance (**Figure 1A**). This suggested that hydralazine dose-dependently increased cell numbers. The results suggested that

incubation with 500 μM hydralazine for 2 h increased the cell number to 8.8 times that of the control (0 μM hydralazine) (**Figure 1A**). The observations using a light microscope (**Figure 1B**) suggested that the cell numbers in each group were similar, suggesting that the MTS method did not accurately estimate the cell numbers.

Similar findings were obtained from VSMCs incubated with hydralazine for 24 h (**Figure 2**). The standard MTS assay suggested that incubation with 500 μM hydralazine for 24 h increased the cell number to 2 times of that of the control (0 μM hydralazine) (**Figure 2A**), whereas incubation with 100 μM hydralazine for 24 h did not affect cell number (**Figure 2A**). These findings contradicted the results from the trypan blue exclusion assay (**Figure 2B**), which suggested that both 100 and 500 μM hydralazine decreased the live cell numbers. The results from the trypan blue exclusion assay were confirmed by observations using a light microscope (**Figure 2C**) which showed that both concentrations of hydralazine (100 and 500 μM) caused cell death.

The direct reaction of MTS with hydralazine in a cell-free system

The possibility of a direct reaction between MTS and hydralazine was then investigated. In the absence of cells, the absorbance of MTS significantly increased in the presence of hydralazine in a dose-dependent manner (**Figure 3**), suggesting the direct reaction of the colorless MTS with hydralazine to produce a colored compound. The reaction was detectable within 2 h and incubation of MTS with hydralazine for 3 h or 4 h did not increase the absorbance further (**Figure 3**).

Modified MTS method can accurately reflect the live cell number of VSMCs incubated with hydralazine

The manufacturer's instruction recommends directly adding MTS to the wells containing cells. Given that hydralazine can directly react with MTS, the assay protocol was modified by replacing the used culture medium in the wells with pre-warmed fresh culture medium immediately before the addition of MTS. The modified MTS assay suggested that incubation of VSMCs with hydralazine for 2 h did not affect the absorbance (**Figure 4**), consistent with observations under the light microscope (**Figure 1B**). Also, the results from the modified MTS assay suggested that incubation of VSMCs with 100 and 500 μM hydralazine for 24 h significantly decreased cells number (**Figure 5**), which was consistent with the results from the trypan blue exclusion assay (**Figure 2B**) and the microscopy observations (**Figure 2C**).

DISCUSSION

This study reported a simple modification of MTS assay that overcame the interference of the testing reagent hydralazine. A discrepancy in the results of the standard MTS assay with those from the trypan blue and microscopic assays was identified. Further investigation discovered that hydralazine directly reacted with the MTS reagent in the absence of cells. A simple modification of the MTS assay protocol, by replacing the used culture medium immediately before the addition of the MTS, was established. The results from the modified MTS assay were in agreement with the results from both the trypan blue exclusion and microscopic assays.

This study used an MTS Cell Proliferation Assay Kit from BioVision Inc., which is a colorimetric method for quantification of viable cells. The method is based on the reduction of MTS tetrazolium compound by viable cells to generate a colored formazan product that is soluble in cell culture media. This conversion is thought to be carried out by NAD(P)H-dependent dehydrogenase enzymes in metabolically active cells[1], in particular mitochondrial succinate dehydrogenase.[14, 15] The formazan dye produced by viable cells can be quantified by measuring the absorbance at 490-500 nm.

MTS can be directly converted into a colored product by hydralazine in a cell-free system in a dose-dependent manner. This reaction completed within 2 h, as an increase in the incubation time further to 3 h or 4 h did not increase the absorbance further. Hydralazine is a reducing reagent that can reduce many oxidizing compounds such as lipid-derived electrophiles[16] and superoxide and peroxynitrite.[17] These findings demonstrate that hydralazine interferes with the MTS assay. It is worthwhile to investigate whether other reducing reagents such as vitamin E and vitamin C can interfere with the MTS assay similarly in the future.

In the presence of the cells, the standard MTS assay suggested that incubation with 50 µM hydralazine for 2 h or 500 µM hydralazine for 24 h increased absorbance (i.e. cell number) which could not be confirmed from the microscopy and trypan blue assays. When cells were incubated with 100 µM or 500 µM hydralazine for 24 h, the trypan blue exclusion assay and microscopic observation suggested that there was reduction in cell numbers compared to the non-hydralazine control, suggesting the cytotoxicity of hydralazine. The false results of the standard MTS assay may be due to the inability of the cultured cells to completely absorb and metabolize the large amounts of hydralazine within 24 h, in contrast to the incubation with 50 µM hydralazine for 24 h. The modified MTS assay replicated the results of the microscopy and trypan blue assay, suggesting it overcame the interference by hydralazine.

MTS assay can be potentially affected by other factors. The assay would be expected to be unreliable when testing compounds that affect mitochondrial function, as mitochondrial succinate dehydrogenase is a key enzyme in the MTS conversion.[14, 15] Indeed, incubation of breast cancer cell line MCF-7 with isopropyl-β-thiogalactosidase has been reported to decrease cell numbers by 60% as measured with trypan blue exclusion assay, however, the MTS assay found an increase by 15% in absorbance.[18] In that study, isopropyl-β-thiogalactosidase incubation increased mitochondrial counts by 3 fold,[18] which is likely responsible for the inaccuracy of the MTS assay.

Reliable measurement of cell viability, proliferation, growth inhibition and death is important in screening for drug treatment efficacy in vitro.[18] To avoid inaccurate result, supplementation of the MTS assay with other non-metabolic assays such as the trypan blue exclusion assay, flow cytometry and microscopic observations is recommended.[19]

In summary, this study suggests an important limitation of the standard MTS assay protocol. A modification of the protocol, where the used culture medium in wells containing cultured

cells is replaced with fresh medium immediately before the addition of MTS reagent, rectifies this problem and results in accurate cell number estimation.

DISCLOSURE STATEMENT

No competing financial interests exist.

FUNDING INFORMATION

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Figure legend

Fig. 1. Discrepancy of live cell numbers of vascular smooth muscle cells (VSMCs) after incubation with hydralazine for 2 h. (**A**) The change in live cell numbers measured by the MTS assay. 200 µL of VSMCs ($1x10^5$ /ml) were placed in each well of a flat-bottom 96-well plate for 24 h. 4 µL of PBS or 4 ul of various concentrations of hydralazine were added to give final concentrations of 0, 10, 50, 100 and 500 µM hydralazine in the wells. 20 µL of MTS was added 2 h later and absorbance was measured using a plate reader 2 h after the addition of MTS. The absorbance value in the well containing the medium plus MTS in the absence of cells was regarded as background and subtracted from the absorbance of other wells. The absorbance in the control group (0 µM hydralazine) was regarded as 100%. N=8. Error bars represent standard deviation. The means among groups were analyzed using one-way ANOVA followed by Bonferroni post-hoc tests. (**B**) Representative images of VSMCs in panel **A** after incubation with hydralazine for 2 h but before the addition of MTS. Scale bar: 25 µm.

Fig. 2. Discrepancy of live cell numbers of VSMCs after incubation with hydralazine for 24 h. (**A**) The change in live cell numbers measured by the MTS assay. 200 µL of VSMCs ($1x10^5$ /ml) were placed in each well of a flat-bottom 96-well plate for 24 h. 4 µL of PBS or various concentrations of hydralazine were added to give final concentrations of 0, 10, 50, 100 and 500 µM hydralazine in the wells. 20 µL of MTS was added 24 h later and absorbance was measured using a plate reader 2 h after the addition of MTS. The absorbance value in the well containing the medium plus MTS in the absence of cells was regarded as background and subtracted from the absorbance of other wells. The absorbance in the control group (0 µM hydralazine) was

regarded as 100%. N=8. (**B**) The change in live cell numbers measured by the trypan blue method. 2 mL of VSMCs ($1x10^5$/ml) were placed in a flat-bottom 6-well plate for 24 h. 4 µL of PBS or various concentrations of hydralazine were added to give final concentrations of 0, 10, 50, 100 and 500 µM hydralazine. 24 h later, the cells were trypsinized and cell numbers were counted using the trypan blue method. The cell number in the control group (0 µM hydralazine) was regarded as 100%. N=4. Error bars represent standard deviation. The means among groups were analyzed using one-way ANOVA followed by Bonferroni post-hoc tests. *P<0.001 compared to all the other groups. (C) Representative images of VSMCs in a 96-well plate after hydralazine incubation for 24 h. Scale bar: 25 µm.

Fig. 3. The reactivity of hydralazine with MTS. 4 µL of PBS or various concentrations of hydralazine were added to 200 µL of cell culture medium without cells in wells of a flat-bottom 96-well plate to give final concentrations of 0, 10, 50, 100 and 500 µM hydralazine. 20 uL of MTS was then added and absorbance was measured using a plate reader 2 h after addition of MTS. The absorbance value in the well containing the medium only was regarded as background and subtracted from the absorbance of other wells. The absorbance of the control group (0 µM hydralazine at 0 h) was regarded as 1 fold (baseline). Error bars represent standard deviation. The means among groups were analyzed using one-way ANOVA followed by Bonferroni post-hoc tests. *P<0.001 compared to any other groups with the same concentration of hydralazine.

Fig. 4. The live cell numbers of VSMCs after incubation with hydralazine for 2 h measured by the modified MTS assay. 200 µL of VSMCs ($1x10^5$ /ml) were placed in each well of a flat-bottom 96-well plate for 24 h. 4 µL of PBS or various concentrations of hydralazine were added

to give final concentrations of 0, 10, 50, 100 and 500 μM hydralazine in the wells. 2 h later, the medium was aspirated and replaced with pre-warm (37C) 200 μl culture medium. 20 μl of MTS was then added and absorbance was measured using a plate reader 2 h after the addition of MTS. The absorbance value in the well containing the medium plus MTS in the absence of cells was regarded as background and subtracted from the absorbance of other wells. The absorbance in the control group (0 μM hydralazine) was regarded as 100%. N=8. Error bars represent standard deviation. The means among groups were analyzed using one-way ANOVA followed by Bonferroni post-hoc tests.

Fig. 5. The live cell numbers of VSMCs after incubation with hydralazine for 24 h measured by the modified MTS assay. 200 μL of VSMCs ($1x10^5$ /ml) were placed in each well of a flat-bottom 96-well plate for 24 h. 4 μL of PBS or various concentrations of hydralazine were added to give final concentrations of 0, 10, 50, 100 and 500 μM hydralazine in the wells. 24 h later, the medium was aspirated and replaced with pre-warm (37C) 200 μl culture medium. 20 μl of MTS was then added and absorbance was measured using a plate reader 2 h after the addition of MTS. The absorbance value in the well containing the medium plus MTS in the absence of cells was regarded as background and subtracted from the absorbance of other wells. The absorbance in the control group (0 μM hydralazine) was regarded as 100%. N=8. Error bars represent standard deviation. The means among groups were analyzed using one-way ANOVA followed by Bonferroni post-hoc tests.

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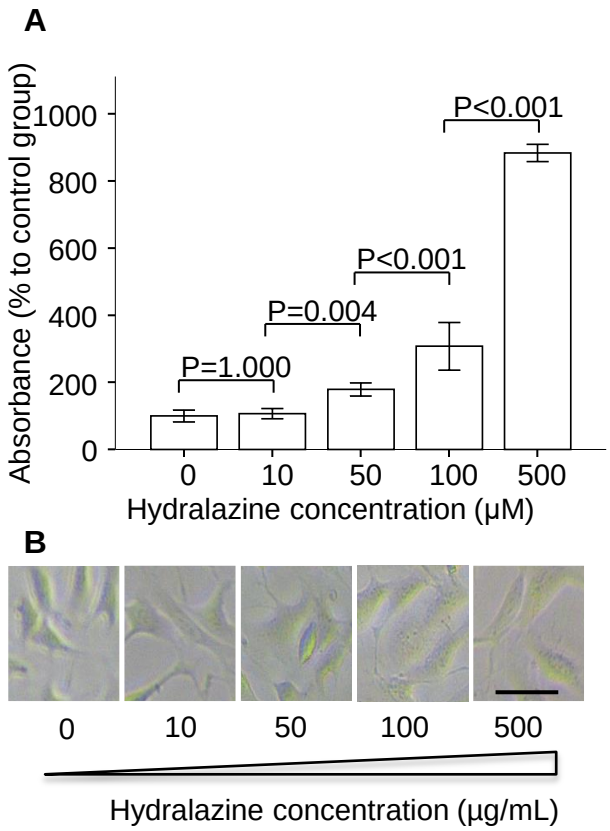
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Absorbance (% to control group)
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800
600
400
200
0
P<0.001
P<0.001
P=0.004
P=1.000
0
10
50
100
500
Hydralazine concentration (μM)
B
0
10
50
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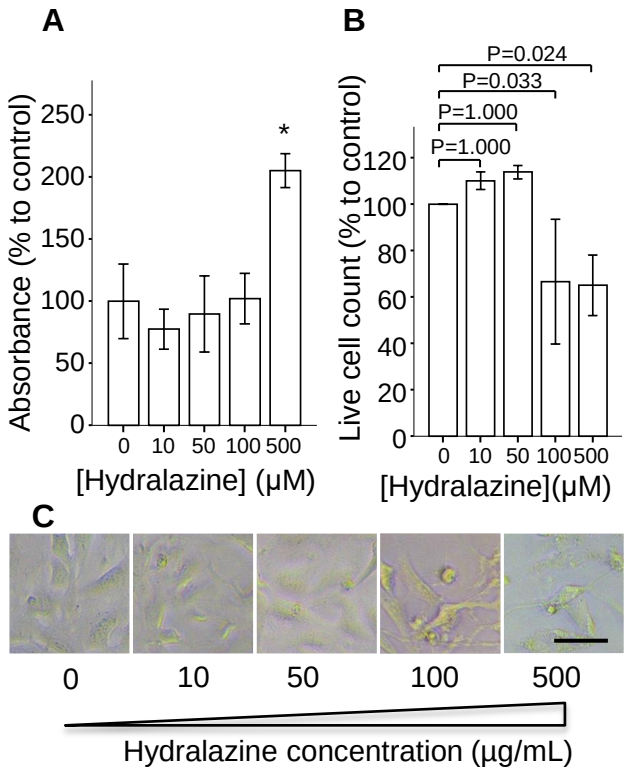
500
Hydralazine concentration (μg/mL)

Figure 1


A
Absorbance (% to control)
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200
150
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50
0
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0 10 50 100 500
[Hydralazine] (μM)
B
Live cell count (% to control)
P=0.024
P=0.033
P=1.000
P=1.000
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60
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[Hydralazine](μM)
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Hydralazine concentration (μg/mL)

Figure 2

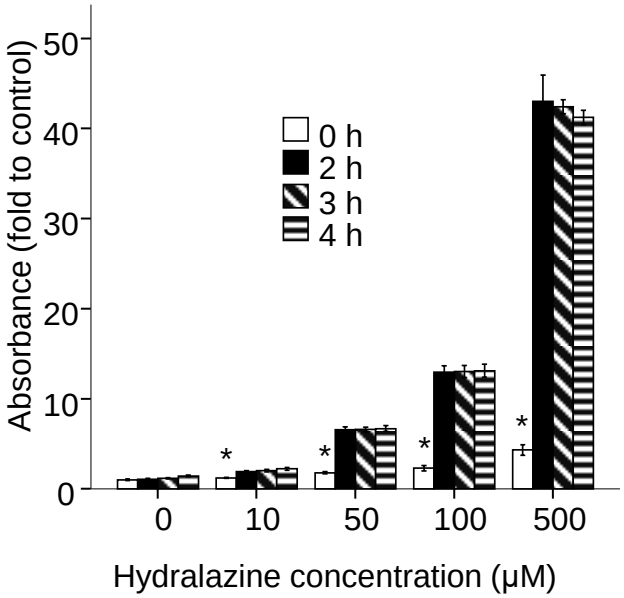


Figure 3

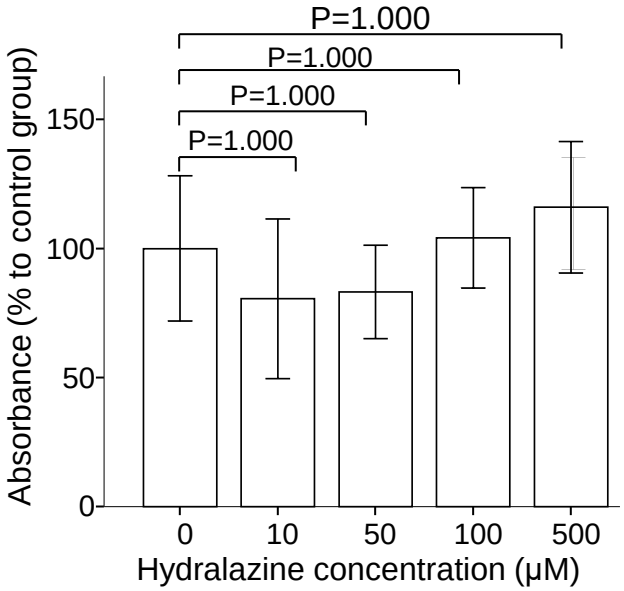
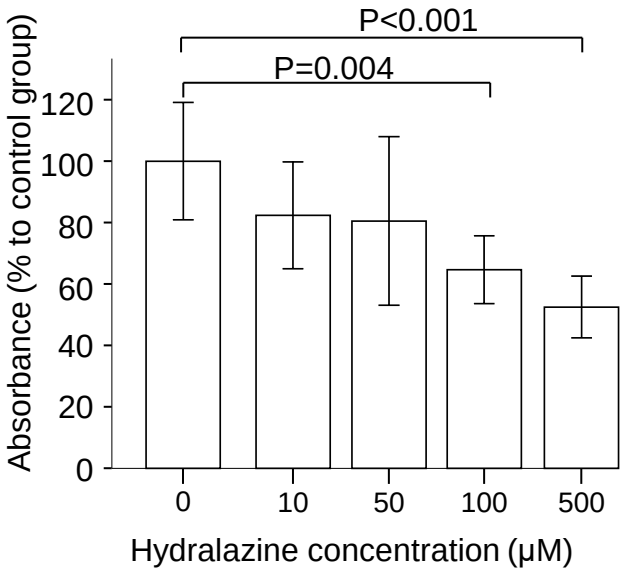


Figure 4


P<0.001
P=0.004
Absorbance (% to control group)
120
100
80
60
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0
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500
Hydralazine concentration (μM)

Figure 5