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Curcumin Attenuates Hippuric Acid-Induced Eryptosis by Enhancing Antioxidant Enzyme Activity

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ABSTRACT

Background: Eryptosis, the programmed death of erythrocytes, is exacerbated under pathological conditions such as chronic kidney disease (CKD), largely due to the accumulation of uremic toxins like hippuric acid. These toxins promote oxidative stress and disrupt erythrocyte membrane integrity, leading to hemolysis and anemia. Curcumin, a polyphenolic compound derived from Curcuma longa, is well known for its potent antioxidant and cyto-protective properties, yet its protective role against uremic toxin-induced eryptosis remains underexplored. **Objective:** This study aimed to investigate the protective effects of curcumin against hippuric acid-induced eryptosis in human erythrocytes and to explore the involvement of oxidative stress and calcium influx in the onset of eryptosis. **Methods:** Human erythrocytes were treated with different concentrations of hippuric acid (300 -430 µM) and curcumin (5-15 µM) for 48 hours. The hemolysis % was determined to assess the cytotoxic effect of hippuric acid. The oxidative potential of hippuric acid and antioxidant potential of curcumin was determining by assessing the activities of antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase. Morphological changes were assessed via mean corpuscular volume (MCV). Amlodipine was used to confirm the role of calcium in eryptosis. **Results:** Hippuric acid significantly increased hemolysis %, reduced MCV, and suppressed activity of antioxidant enzymes (SOD, GPx, and CAT). Curcumin co-treatment markedly attenuated hemolysis %, restored MCV, and significantly upregulated antioxidant enzyme activity. Amlodipine pretreatment effectively blocked calcium channels thus mitigated the effect of hippuric acid. **Conclusion:** Curcumin protects erythrocytes from hippuric acid-induced eryptosis by restoring antioxidant defenses and providing cyto-protective properties. These findings reveal the protective mechanisms of curcumin and suggest its potential utility as a natural therapeutic agent to mitigate erythrocyte damage in CKD and related oxidative stress conditions.

INTRODUCTION

Erythrocytes, commonly known as red blood cells, are the most important component of human blood [1]. Mature mammalian erythrocytes are anucleated cells [2]. They have unique disc-like biconcave shape with a diameter of almost 8 µm, which distinguishes them from other cell types not only in morphology but also in function. This unique shape imparts remarkable flexibility, enabling them to transverse freely across the narrow capillaries and to efficiently carry out gaseous and nutrient exchange [3]. They also play a vital role in maintaining acid-base equilibrium of blood and body [4].

Hematopoiesis is a continuous process of production of blood cells. It involves differentiation, proliferation and maturation of cells [5]. Different stages of this process occurs in different locations in the body. Erythropoiesis, a part of hematopoiesis, is a complex process of synthesis of erythrocyte that starts in bone marrow and ends in peripheral blood circulation. Through this process, the body of an adult human being generates approximately two million erythrocytes per second [6]. This process takes place in three stage: differentiation of progenitors, maturation of erythroblasts and formation of reticulocytes [7]. Myeloid progenitors develop into erythroblasts while undergoing morphological changes and hemoglobin synthesis then eventually after expulsion of nucleus they develops into reticulocytes. These reticulocytes when ejected into the blood stream matures into erythrocytes

and are allowed to move freely for upto 120 days and then they are removed by macrophages [8].

Eryptosis, a programmed suicidal cell death of erythrocytes, happens when an erythrocyte is exposed to injury or harmful materials. It is carried out to remove damaged erythrocytes from blood circulation [9]. The process of eryptosis is triggered by energy depletion, exposure to xenobiotics, osmotic shock, heavy metals and oxidative stress [10]. Oxidative stress is the state in the body in which there is imbalance between the production of oxidants and antioxidants along with suppressed degradation of harmful radicals [11, 12]. This leads to cellular and molecular damage as the free radicals like ROS and RON can disrupt lipids and proteins of erythrocyte's cell membrane [13]. Oxidative stress is linked with onset of many inflammatory diseases especially chronic kidney disease [14]. In erythrocytes, the oxidative stress causes lipid peroxidation and protein cross-linking leading to hemolysis and apoptosis via activation of caspases [15, 16].

Hippuric acid, a protein bound uremic toxin, is involved in advancement in CKD and renal fibrosis [17]. It disrupts the normal redox balance and causes fibrosis of kidney [18]. Hippuric acid also accelerates the imbalance in the extracellular matrix of cells and the expression of fibrosis-linked genes. The hippuric acid by lowering the levels of nuclear factor erythroid 2-related factor 2 (NRF2) imbalances the anti-oxidant system and thus stimulates renal fibrosis in the patient with CKD [17]. In the patients of CKD, the value of hippuric acid is significantly higher than the other protein-bound uremic toxins [19].

Antioxidants are those substances that prevent or control the process of oxidation only when they are in low concentration in the body or in food [20]. They also prevent the progression and onset of degenerative diseases in the body. Erythrocytes are protected against oxidative damage by enzymatic antioxidants such as catalase (CAT) and superoxide dismutase (SOD) [2] and non-enzymatic antioxidants such as reduced glutathione (GSH). SOD converts reactive H_2O_2 to O_2 [21]. Catalase removes intra-cellular and extra-cellular H_2O_2 [22]. GSH protects proteins and lipids of the membrane thus stabilizes the membrane of erythrocytes. Primary antioxidant defense is provided by them to erythrocytes [2]. It also acts as sulfhydryl buffer to maintain reduce state of SH groups in enzymes and Hb [19].

Curcumin is a phenolic compound obtained from turmeric/Curcuma longa and is used as a spice, food additive and medicinal herb in Ayurvedic and Chinese medicine [23]. Curcumin exhibits direct and indirect antioxidant activities. Directly it scavenges radical species such as nitric oxides and superoxide anions [24]. Indirectly it acts as an activator of Nrf2 pathways that enhances the production of anti-oxidant enzymes and cyto-protective proteins such as glutathione S-transferase, SOD, and CAT [25]. Curcumin also exhibits anti-inflammatory properties by inhibiting the activation of c-Jun N-terminal kinase (JNK) and NF-κB pathways [26]. It also rectifies the gut barrier, reduces the risk of CKD-induced CVDs, and lowers the kidney damage by decreasing the glomerulosclerosis index [27]. It regulates different biological processes such as the phosphatidylinositol 3-kinase pathway, expression of proteins, production of ceramides, arrangement of chromosomes, eicosanoid formation, and telomerase [28]. Curcumin can precisely target signal transduction pathways like NF-kB and Nrf-2 pathways that are involved in inducing oxidative stress and inflammation [29]. Thus it can become a potent alternative for treatment therapies. In the present study, we aim to determine the eryptotic, hemolytic and oxidative potential of hippuric acid, to investigate the anti-eryptotic potential of curcumin in hippuric acid induced eryptosis and to verify the role of calcium in the onset of eryptosis. To our best knowledge, it is the first study to determine the protective properties of curcumin in hippuric acid-induced eryptosis on human erythrocytes.

MATERIALS AND METHODS

After the approval of the Directorate of Graduate Studies, University of Agriculture, Faisalabad experimental work was carried out. From different blood banks of Faisalabad, screened blood samples were obtained and were persevered in heparin tubes till further use to prevent hemolysis.

Isolation of Erythrocytes

Blood samples were centrifuged at 1500 rpm for 5 minutes to separate plasma. Washing of blood was carried out by adding 2 ml of ringer solution to the centrifuged sample. Then again centrifugation was carried out at 1500 rpm for 5 minutes, supernatant was removed using a micro-pipette and erythrocytes were obtained [30].

Preparation of Sample

Using isolated erythrocytes, two types of samples namely A and B were prepared. Sample A included 1 control (C) and 3 treatments (T_1, T_2, T_3). C included 1000 µL of ringer solution along with 4 µL of blood. T_1, T_2, T_3 included 1000 µL of ringer solution, 4 µL of blood. 1 µL of various concentrations of Hippuric acid such as 300 µM, 398 µM and 430 µM are added in T_1, T_2, T_3 respectively.

In type B of samples, 2 controls (C and C^+) and 3 treatments (T_1, T_2, T_3) were used. Control 1 (C) included 1ml ringer, 4 µL blood and control B (C^+) contained 1ml ringer, 4 µL blood, and 1 µL of 398 µM Hippuric acid. The treatments included 1ml ringer, 4 µL blood, and 1 µL of 398 µM Hippuric acid and then were treated with various concentrations of phytochemical curcumin i.e. 5µM, 10µM and 15µM.

Measurement of Hemolysis Percentage

Incubation of both types of sample was done at 37°C for 48 hours. After 48 hours, samples were micro-centrifuged at 3000 rpm for 3 minutes. Supernatants that have hemoglobin were collected for the measurement of hemolysis % in a spectrophotometer at 571 nm [31].

Measurement of Oxidative Stress

Different antioxidant systems are already present inside the erythrocytes to counter the effect of ROS that are formed in it. The human body possesses many anti-oxidant enzymes such as glutathione peroxidase (GP_x), superoxide dismutase (SOD), and Catalase (CAT), carrier proteins, trapping molecules, and pharmacological defense system. The defense molecules include some vitamins such as vitamins A and D, and glutathione (GSH). Moreover, many

phytochemicals i.e. chrysin, resveratrol, curcumin, and allicin etc. have anti-oxidant activities that can be used to reduce oxidative stress in cells.

Superoxide Dismutase (SOD)
The antioxidant potential of the cell was determined by measuring the activity of SOD. Riboflavin and methionine are mixed to generate superoxide radical O_2^- which either reduces NBT (a yellow chromophore to blue precipitates) or gets oxidized by SOD. For the determination of SOD activity in both types of samples, the Elisa plate reader was used at 570 nm [30]. The lower the photo reduction potential of NBT, the higher the activity of SOD.

The Reaction Mixture of Superoxide Dismutase (SOD)
The reaction mixture components of SOD were made as follow: mixed 0.03g of NBT in 30 ml of distilled water, 0.44g of methionine in 30 ml of distilled water, 0.035g of Triton X-100 in 15ml of distilled water, 0.035g of riboflavin in 15ml of distilled water. Then dissolving 100 µL of NBT, 200 of µL methionine, 200 µL of Triton X-100, and 500 µL of phosphate buffer in 800 µL distilled water [31].

Assay Procedure for SOD
A 96-welled microtiter plate was taken and 50 µL of samples and 50 µL of the reaction mixture of SOD is loaded in it. Then kept it under the UV lamp for 15 minutes. After 15 minutes added 25 µL riboflavin and mixed it. Then placed these plates in an ELISA plate reader and took absorbance at 570 nm. The same procedure was used for both samples A and B [30].

Glutathione Peroxidase (GPx)
In the presence of glutathione peroxidase H_2O_2 is converted into H_2O and O_2. The oxygen reacts with guaiacol and produces brown colored compound whose absorbance is measured in the Elisa plate reader.

The Reaction Mixture of Glutathione Peroxidase (GPx)
The reaction mixture of POD was formed by mixing 100 µL of 20 mM Guaiacol, 100 µL of 40 mM H_2O_2, and 400 µL of 0.2 M phosphate buffer [31].

Assay Procedure for POD
In the 96-welled microtiter plate 50 µL of samples and 50 µL of the POD reaction mixture were loaded, then this plate was placed in an ELISA plate reader and absorbance at 490 nm was taken [30]. The same procedure was carried out for both samples A and B.

Catalase
H_2O_2 was converted to H_2O and O_2 by catalase enzyme and absorbance was measured in the Elisa plate reader.

The Reaction Mixture of CAT
The reaction mixture of CAT is made by mixing 100 µL of H_2O_2 and 100 µL phosphate buffer [31].

Assay Procedure for CAT
50 µL of samples and 50 µL of the reaction mixture of CAT were loaded in different wells of 96 well microtiter plates which is then placed in an ELISA plate reader for taking absorbance at 240 nm [30]. Followed the same procedure for samples A and B.

Measurement of MCV (Mean Corpuscular Volume)
The main features of an erythrocyte that has undergone eryptosis are shrinkage and membrane blebbing. An automated hematology analyzer was used to determine the mean corpuscular volume [31].

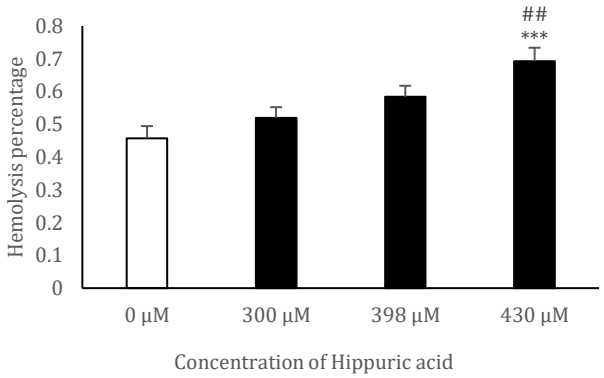
Assessment of Ca^{2+} Channel Activity
Amlodipine is a potent blocker of the Ca^{2+} channel. When erythrocytes were treated with toxins in the presence of Ca^{2+} they showed eryptosis. Erythrocytes were then treated with toxins in presence of amlodipine. The activity of the Ca^{2+} channel was confirmed using an antioxidant enzyme assay [31].

Statistical Analysis
The experiments were carried out using almost 2 to 4 treatments in 12 repeats. Statistical analysis was carried out using one-way ANOVA and mean comparison was calculated using Tukey's test [31] and GraphPad InStat 3 software.

RESULTS

Measurement of Hemolysis Percentage
Hemolysis is an unprogrammed process of destruction of erythrocytes in which cellular components is released in the surrounding environment. When erythrocytes are given stress such as oxidative stress, they initiate hemolysis that further starts eryptosis. The induction of hippuric acid imposes oxidative stress thus leads to increased hemolytic index.

Figure 1
Variations in Hemolysis % due to Hippuric Acid



Arithmetic means ±SEM (n = 12) were determined. Fig. 4.1 shows that the graph was plotted by taking the different concentrations of hippuric acid along X-axis and the hemolysis percentage along Y-axis. The white bar indicates untreated sample whereas the black bars indicate different concentrations of hippuric acid (300 µM, 398 µM, and 430 µM). Hippuric acid induced eryptosis in erythrocytes which was determined by the bursting of the cell and leakage of hemoglobin from the cells. The hemolysis percentage of the treated cells was determined. The significant variation in treated and non-treated sample is shown by *** ($p<0.001$) and variation within the treated sample is shown by ## ($p<0.01$) showed significant variation within treatments. The results have shown that by increasing the concentration of hippuric acid, the hemolysis % will also increase.

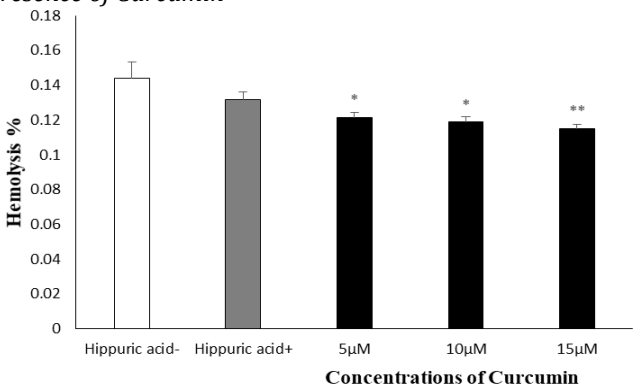
Measurement of Hemolysis % due to Hippuric Acid in the Presence of Curcumin
As the process of eryptosis is linked with hemolysis so the

erythrocytes were treated with a single reported concentration of hippuric acid and three different concentrations of curcumin.

Curcumin is a known antioxidant and anti-inflammatory compound. Its presence will hinder the process of hemolysis as it is a potent scavenger of free radicals and protects erythrocytes against oxidative damage as discussed by [24].

Figure 2

Variation in Hemolysis % due to Hippuric Acid in the Presence of Curcumin



Arithmetic means ±SEM (n = 12) were determined. Fig. 4.2 shows that the graph was plotted by taking the different concentrations of curcumin along X-axis and the hemolysis percentage along Y-axis. The Hippuric acid$^-$ (white bar) indicates negative control which is without toxin and curcumin and Hippuric acid$^+$ (grey bar) indicates positive control which includes hippuric acid along with blood and ringer solution. The black bars indicate different concentrations of curcumin (5 µM, 10 µM, and 15 µM). The noteworthy variations in treated and non-treated samples are shown by * ($p<0.05$) and ** ($p<0.01$).

The results have shown that by increasing the concentration of curcumin, the hemolysis % will decrease. This result is in accordance with the results obtained by [32] in his study. Almost the same results of reducing cell death by curcumin were also reported in [33]. Same results of decrease in hemolysis % of erythrocytes using curcumin were also reported by [34] in their study.

These results showed the remarkable ability of curcumin to counter the effect of hippuric acid by lowering the rate of hemolysis and cell death of erythrocytes.

Measurement of Oxidative Stress

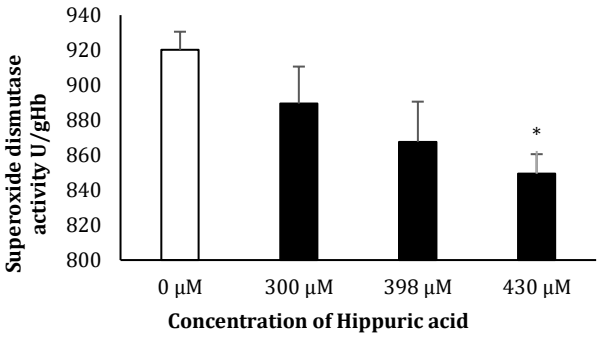
Oxidative stress is the one of main culprit behind the stimulation of eryptotic pathways. Hippuric acid produces oxidative stress and curcumin counters this effect of hippuric acid by enhancing the activity of antioxidant enzymes such as SOD, POD and CAT that can be assayed using Elisa plate reader.

Superoxide Dismutase

SOD is the one of most important enzyme that act as front line defensive enzyme against oxidative stress. It accelerates the dismutation of superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen (O_2). Erythrocytes were treated with different concentrations of hippuric acid (0 µM, 300 µM, 398 µM, and 430 µM) and the activity of SOD was measured using ELISA plate reader.

Figure 3

Variations in Superoxide Dismutase (SOD) Activities (U/g Hb) due to Hippuric Acid



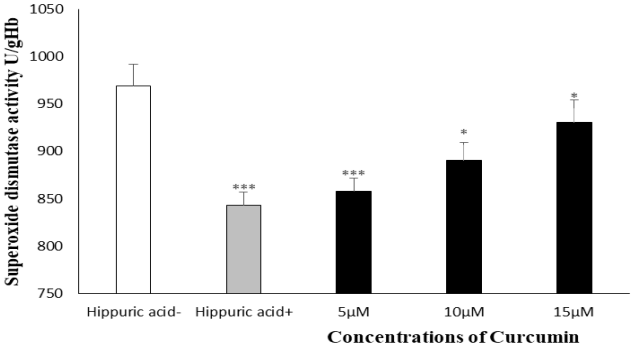
Arithmetic means ±SEM (n = 12) were determined. Fig 4.3 shows that the graph was plotted by taking the different concentrations of hippuric acid along the X-axis and the activity of superoxide dismutase (SOD) along the Y-axis. The white bar indicates untreated sample that lacks hippuric acid whereas the black bars indicate different concentrations of hippuric acid (300 µM, 398 µM, 430 µM). The enzymatic activity of both control and treatments was determined. The remarkable variation between treated and non-treated samples is shown by * ($p<0.05$). The results have shown that by increasing the concentration of hippuric acid, the activity of SOD will decrease. Hippuric acid disrupted the normal redox balance that causes the decrease in SOD's activity. Similar results of decreased SOD activity in the presence of hippuric acid were reported by [17] and [35] in their studies.

Measurement of Superoxide Dismutase Activity (SOD) due to Hippuric Acid in the Presence of Curcumin

To study the antioxidant effect of curcumin in the presence of hippuric acid, the erythrocytes were treated with a single reported concentration of hippuric acid and three different concentrations of curcumin that are 5 µM, 10 µM, and 15 µM. Then the activity of SOD was measured using ELISA plate reader.

Figure 4

Variations in Superoxide Dismutase (SOD) Activities (U/g Hb) due to Hippuric Acid in the Presence of Curcumin



Arithmetic means ±SEM (n = 12) were determined. Fig. 4.4 shows that the graph was plotted by taking the different concentrations of curcumin along the X-axis and the activity of superoxide dismutase along the Y-axis. Hippuric acid$^-$ (white bar) indicates negative control which is without toxin and curcumin and Hippuric acid$^+$ (grey bar)

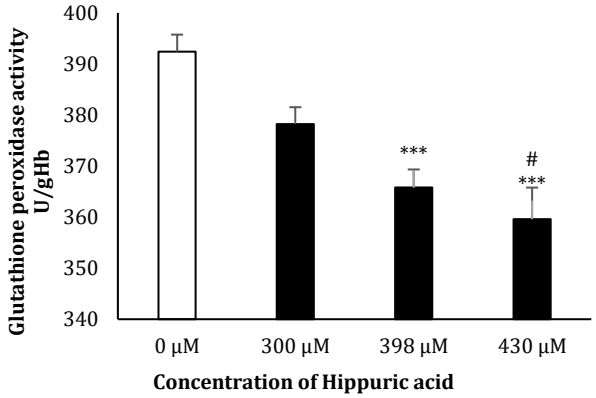
indicates positive control which includes hippuric acid along with blood and ringer solution. The black bars indicate different concentrations of curcumin (5 µM, 10 µM, and 15 µM). The significant variations in treated and non-treated samples are shown by *** ($p<0.001$) and * ($p<0.05$). The results have shown that by increasing concentration of curcumin, an increase in the activity of SOD was observed. This increase in the activity of the SOD enzyme is due to an increase in the free radical scavenging ability of superoxide dismutase. The results of my study is similar to the results of [36], [37] and [14].

Glutathione Peroxidase (GPx)

GPx is an antioxidant enzyme that degrades the hydrogen peroxide which is a toxic compound for cell to its less or non-toxic form. It reduces H_2O_2 into water by oxidizing the reduced glutathione (GSH) into its disulfide/ oxidized form GSSH. Its activity is measured by observing the change in colour that happens due to presence of substrate like guaiacol. Erythrocytes were treated with different concentrations of hippuric acid (0 µM, 300 µM, 398 µM, and 430 µM) and the activity of peroxidase enzyme was measured.

Figure 5

Variations in GPx Activities (U/g Hb) due to Hippuric Acid



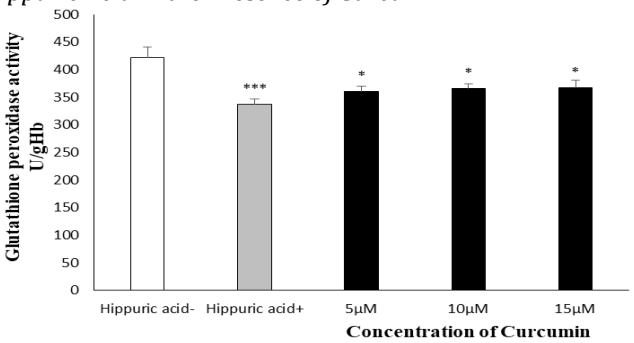
Arithmetic means ±SEM (n = 12) were determined. Fig 4.5 shows that the graph was plotted by taking the different concentrations of hippuric acid along the X-axis and the activity of glutathione peroxidase along the Y-axis. The white bar indicates untreated sample that lacks hippuric acid whereas the black bars indicate different concentrations of hippuric acid (300 µM, 398 µM, and 430 µM). The enzymatic activity of both untreated and treated samples was determined. The noteworthy variation between treated and non-treated samples is shown by *** ($p<0.001$). Whereas the variation within the treated samples is shown by # ($p<0.05$). The results have shown that by increasing the concentration of hippuric acid, the activity of glutathione peroxidase will decrease. These results are consistent with earlier findings of decreased GPx activity in the presence of hippuric acid and were reported by [17], [35] and [38] in their studies.

Measurement of Glutathione Peroxidase Activity (GPx) due to Hippuric Acid in the Presence of Curcumin

Just like SOD, curcumin also enhances the activity of GPx. The phenolic antioxidants like curcumin has benzene structure with hydroxyl group. The ROS disrupts the electrons of phenol ring thus make them best scavengers of radical species. The erythrocytes were treated with a single reported concentration of hippuric acid and three concentrations of curcumin that are 5 µM, 10 µM, and 15 µM. Then the activity of GPx was measured.

Figure 6

Variation in Activity of Glutathione Peroxidase due to Hippuric Acid in the Presence of Curcumin



Arithmetic means ±SEM (n = 12) were determined. Fig. 4.6 shows that the graph was plotted by taking the different concentrations of curcumin along the X-axis and the activity of glutathione peroxidase along the Y-axis. Hippuric acid$^-$ (white bar) indicates negative control which is without toxin and curcumin and hippuric acid$^+$ (grey bar) indicates positive control which includes hippuric acid along with blood and ringer solution. The black bars indicate different concentrations of curcumin (5 µM, 10 µM, and 15 µM). The significant variations in treated and non-treated samples are shown by *** ($p<0.001$) and * ($p<0.05$). The results have shown that by increasing the concentration of curcumin, an increase in the activity of GPx was observed.

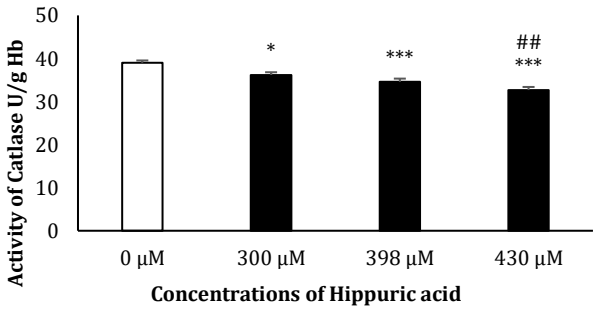
Analogous results of increased GPx activity in the presence of curcumin were reported by [36], [34], [37] and [14] in their studies.

Catalase

Catalase (CAT) is an antioxidant enzyme that uses hydrogen peroxide as its substrate. This enzyme neutralizes non radical ROS such as H_2O_2 into H_2O thus maintains the normal physiological conditions within cell that are necessary for signal transductions. Erythrocytes were treated with different concentrations of hippuric acid (300 µM, 398 µM, and 430 µM) and the activity of CAT was measured.

Figure 7

Variations in Catalase Activities (U/g Hb) due to Hippuric Acid



Arithmetic means ±SEM (n = 12) were determined. Fig. 4.7 shows that the graph was plotted by taking the different concentrations of hippuric acid along the X-axis and the activity of catalase enzyme along the Y-axis. The white bar indicates untreated sample that lacks hippuric acid whereas the black bars indicate different concentrations of hippuric acid (300 µM, 398 µM, and 430 µM). The enzymatic activity of both treated and untreated sample was determined. The remarkable variations between treated and non-treated samples are shown by * ($p<0.05$) and *** ($p<0.001$). Whereas the variation within the treated samples is shown by ## ($p<0.01$). The results have shown that by increasing the concentration of hippuric acid, the activity of the catalase enzyme will decrease.

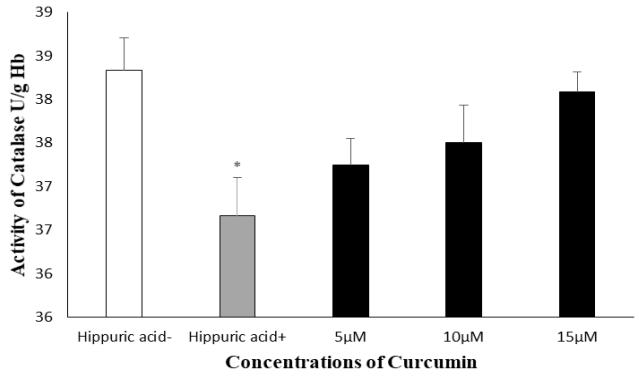
Untreated sample (white bar) showed maximum catalase (CAT) activity as it lacked hippuric acid which is a uremic toxin. Whereas treated cells (black bars) effectively reduced the activity of CAT. These results of decreased CAT activity correspond to the results of [17], [39] and [40] in their studies.

Measurement of Activity of Catalase (CAT) due to Hippuric Acid in the Presence of Curcumin

Erythrocytes were treated with a single reported concentration of hippuric acid and 3 concentrations of curcumin and the activity of CAT was measured.

Figure 8

Variation in Activity of Catalase due to Hippuric Acid in the Presence of Curcumin



Arithmetic means ±SEM (n = 12) were determined. Fig 4.8 shows that the graph was plotted by taking the different concentrations of curcumin along the X-axis and the activity of catalase (CAT) along the Y-axis. Hippuric acid$^-$ (white bar) indicates negative control which is without toxin and curcumin and Hippuric acid$^+$ (grey bar) indicates positive control which includes hippuric acid along with blood and ringer solution. The black bars indicate different concentrations of curcumin (5 µM, 10 µM, and 15 µM). The noteworthy variation in treated and non-treated samples is shown by * ($p<0.05$). The results have shown that by increasing the concentration of curcumin, an increase in the activity of catalase (CAT) was observed.

Results indicate that the activity of catalase (CAT) which is an anti-oxidant enzyme increases by increasing the concentration of curcumin which is a strong anti-oxidant compound. Hippuric acid$^-$ (negative control) showed maximum catalase activity as it lacked hippuric acid which is a strong uremic toxin. Hippuric acid$^+$ (grey bar) which indicates positive control showed minimum catalase activity as it contained hippuric acid without curcumin. Whereas treated cells (black bars) showed an increase in catalase activity with the increase in the concentration of curcumin. This increase in the activity of the catalase enzyme is due to the increase in the free radical scavenging ability of catalase which is made possible due to the induction of curcumin.

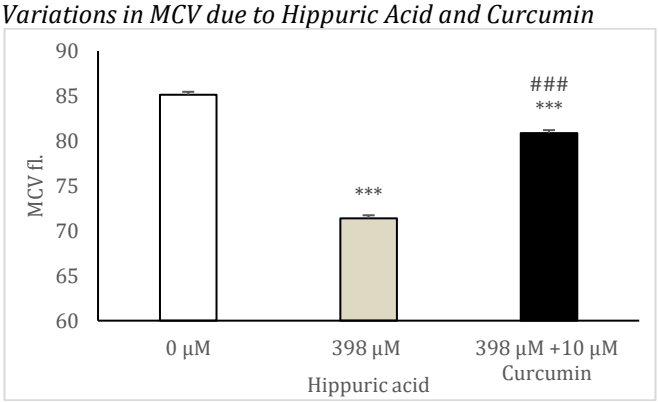
Similar results of increased activity of CAT in the presence of curcumin were reported by [36], [37] and [14].

Measurement of Erythrocyte's Mean Corpuscular Volume (MCV)

Erythrocytes are very sensitive with regard to their membrane structure and integrity. Any change in cell size and volume indicates the presence of stress molecule or any other endogenous harmful substance. MCV of erythrocytes was determined using a hematology analyzer. Because of the treatment of hippuric acid on erythrocytes, oxidative stress started and the size of erythrocytes got mutated.

Figure 9

Variations in MCV due to Hippuric Acid and Curcumin



Arithmetic means ±SEM (n = 12) were determined. Fig 4.9 shows that the erythrocytes were treated with 398 µM hippuric acid and 10 µM Curcumin and then incubated for 24 hours. The graph was plotted by taking hippuric acid concentrations along the X-axis and the MCV along the Y-axis. White bar is the untreated sample of erythrocytes that lacks both hippuric acid and curcumin, the grey bar indicates erythrocytes treated with hippuric acid and the black bar indicates erythrocytes treated with curcumin. The noteworthy variation between treated and non-treated samples was shown by *** ($p<0.001$) and the variation between treated samples is shown by ### ($p<0.001$).

The results showed that the MCV decrease when the erythrocytes were treated with hippuric acid while a rise in MCV was observed in samples that were treated with curcumin. These results showed that induction of hippuric acid leads to shrinkage of cells thus causing eryptosis whereas induction of curcumin prevents shrinkage of erythrocytes thus ultimately inhibiting eryptosis.

Cytoprotective property of curcumin is quite evident from the results mentioned above in Fig 4.9. Similar evidences of cytoprotective property of curcumin were well documented in literature by [41] and [42].

Role of Calcium (Ca^{2+}) in the Stimulation of Eryptosis

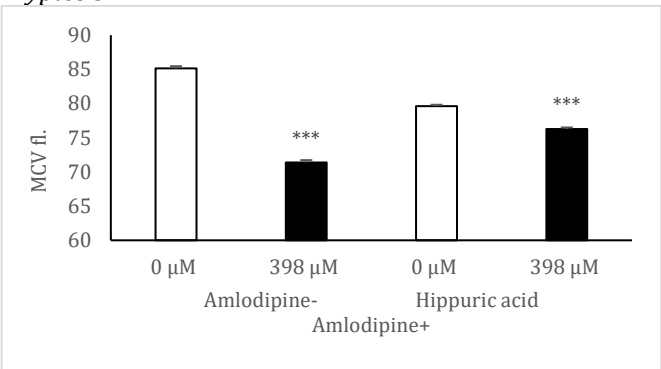
Ca^{2+} is the main culprit behind the stimulation of eryptosis. The intracellular level of Ca^{2+} got disrupts due to the

oxidative stress. OS stimulates the entry of Ca^{2+} into the erythrocytes that further activates Ca^{2+}-dependent K^+ channels. This influx of Ca^{2+} causes scrambling of PS, blebbing and ultimately shrinkage of cell. Amlodipine is a strong Ca^{2+} blocker. It is used to treat high blood pressure and other CVDs.

Some samples were treated with a single reported concentration of hippuric acid and some were left untreated. Then samples were divided into two equal groups. One group was given amlodipine while the other group was not given it. After this, samples were incubated for 24 hours and their MCVs were calculated. All this was done to determine the role of Ca^{2+} in the stimulation of eryptosis. The change in their MCV indicates the stimulation of eryptosis.

Figure 10

Confirmation of Role of Ca^{2+} in Hippuric Acid Stimulated Eryptosis



Arithmetic means ±SEM (n = 12) were determined. The erythrocytes were treated with 398 µM hippuric acid and then incubated for 24 hours. Fig 4.10 shows that the graph was plotted by taking hippuric acid along the X-axis and the MCV along the Y-axis. White bars are the untreated sample of erythrocytes, and the black bars indicate erythrocytes treated with hippuric acid. The noteworthy variations between treated and non-treated samples were shown by *** ($p<0.001$).

Fig 4.10 shows no significant decrease in MCV in hippuric acid treated and non-treated erythrocytes in the presence of amlodipine. These results imply that amlodipine effectively blocked Ca^{2+} channels and hippuric acid was unable to increase intracellular Ca^{2+} level which was the cause of cell shrinkage and eryptosis. Similar results of blockage of Ca^{2+} channels by amlodipine were reported by [43], [44] and [31, 45]. My results were similar to their results.

DISCUSSION AND CONCLUSION

This study provides strong evidence that Curcumin, a natural compound in turmeric can significantly protects erythrocytes from hippuric acid induced eryptosis. Induction of hippuric acid to erythrocytes significantly disrupts natural redox balance reducing antioxidant activities of SOD, GPx and CAT, increases hemolytic index, disrupts morphology of cell causing cell shrinkage and increased calcium influx. However, treatment with Curcumin significantly counters all aforementioned abnormalities and put forward a strong anti-oxidant and cytoprotective role. Curcumin restored the redox balance and stabilizes the cell membrane.

Oxidative stress happens when the production of ROS overwhelms the antioxidant defense of cell. This causes structural and functional damage. In our study, erythrocytes when treated with hippuric acid exhibited high hemolytic percentages, indicating disruption of cellular membrane under oxidative stress. This oxidative damage was countered and mitigated by curcumin in a dose dependent manner. These findings confirm the role of curcumin in mitigating hippuric acid toxicity stimulated eryptosis. Although it is the first study conducted on human erythrocytes to assess the role of curcumin in countering hippuric acid stimulated eryptosis but similar results of mitigating hippuric acid stimulated eryptosis by curcumin were present in other studies [36], [37] and [14]. Furthermore, our results showed that the activity of antioxidant enzymes – superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) get significantly lowered on treating with hippuric acid but curcumin not only restored but also enhanced the activities of these antioxidant enzymes. This indicates that curcumin not only scavenges free radicals but also helps in strengthen body's very own antioxidant defense system. Moreover, our results also show an increases in mean corpuscular volume (MCV) in curcumin treated sample which demonstrated cytoprotective properties of curcumin. This cytoprotective property of curcumin is responsible for prevention of eryptosis as it prevents shrinkage of cells.

Another important aspect of this study was to verify the role of calcium in the stimulation of eryptosis induced by hippuric acid. When a calcium channel blocker, amlodipine, was introduced to erythrocytes the results showed that there was no significant decrease in the MCV of cells. This outcome showed that amlodipine effectively blocked the calcium channels that were the target of hippuric acid thus no increase in intracellular Ca^{2+} level happens that was the cause of the onset of eryptosis.

In nutshell, the results indicate that Curcumin is a potent phytochemical that protects erythrocytes from the oxidative damage. It exhibits both antioxidant and cytoprotective properties, thus counters ROS, maintain redox balance, maintain integrity and morphology of cell and enhances the antioxidant activity of natural antioxidant enzymes. This makes curcumin a promising compound for treating disorders related to oxidative stress. Although our in vitro data are encouraging, yet future studies need to be focused on determining the molecular pathways being involved, testing the efficacy of curcumin in in-vivo settings and determining pharmacokinetics.

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