Quercetin educes hydroxyurea induced cytotoxicity in Immortalized mouse aortic endothelial cells

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Background Chronic inflammation is a characteristic of sickle cell disease (SCD), and is invariably associated with vascular endothelial injury. Hydroxyurea (HU), a naturally cytotoxic chemotherapeutic agent, is the only FDA drug approved for SCD, and is therefore naturally cytotoxic. Quercetin (QCT) is a dietary flavonoid found ubiquitously in plants and foods that have anti-oxidative and anti-inflammatory characteristics. Our hypothesis is that dietary QCT will decrease cytotoxic effects of lipopolysaccharide (LPS) and HU induced vascular cell damage. LPS was used to induce an inflammatory response in immortalized mouse aortic endothelial cells (iMAECs), representing an *in vitro* model of inflamed vascular endothelium.

Methods Lipopolysaccharide (LPS) was used to induce inflammation in immortalized mouse aortic endothelial cells (iMAECs), providing an in vitro model of inflamed endothelial cells. The cells were exposed to LPS throughout the entire experiment. Interventions included treating the LPS exposed cells with QCT, HU, or QCT + HU over 50 hours. The 50-hour period included 24 hours of varying treatments, followed by two hours of hypoxic exposure and then 24 hours under normal aerobic exposure.

Results LDH level was significantly higher for LPS treated versus untreated cells (P = 0.0004). LPS plus 30 micromole QCT reduced the LDH (p = 0.1, trend), whereas LPS plus 100 micromoles HU, significantly increased LDH (p = 0.0004). However, LPS plus treatment with 30 micromoles QCT/100 micromoles HU, significantly reduced LDH, compared with HU alone (p = 0.0002).

Discussion These results suggest that quercetin may be effective against vascular endothelial cell damage for iMAECs *in vitro*. In particular, it shows promise in preventing HU-induced cytotoxicity, surprisingly found from these results. This latter finding is interesting, and should be given more consideration, since HU is the only FDA-approved drug for treating sickle cell patients, and its use is rapidly increasing.

2 **Quercetin Reduces Hydroxyurea Induced Cytotoxicity in Immortalized Mouse Aortic**

3 **Endothelial Cells**

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15   **Abstract**

17   **Background**

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20   with vascular endothelial injury. Hydroxyurea (HU), a naturally cytotoxic chemotherapeutic

21   agent, is the only FDA drug approved for SCD, and is therefore naturally cytotoxic. Quercetin

22   (QCT) is a dietary flavonoid found ubiquitously in plants and foods that have anti-oxidative and

23   anti-inflammatory characteristics. Our hypothesis is that dietary QCT will decrease cytotoxic

24   effects of lipopolysaccharide (LPS) and HU induced vascular cell damage. LPS was used to

25   induce an inflammatory response in immortalized mouse aortic endothelial cells (iMAECs),

26   representing an *in vitro* model of inflamed vascular endothelium.

27

28   **Methods**

29

30   Lipopolysaccharide (LPS) was used to induce inflammation in immortalized mouse aortic

31   endothelial cells (iMAECs), providing an in vitro model of inflamed endothelial cells. The cells

32   were exposed to LPS throughout the entire experiment. Interventions included treating the LPS

33   exposed cells with QCT, HU, or QCT + HU over 50 hours. The 50-hour period included 24

34   hours of varying treatments, followed by two hours of hypoxic exposure and then 24 hours under

35   normal aerobic exposure.

36

37   **Results**

38   LDH level was significantly higher for LPS treated versus untreated cells ($P = 0.0004$). LPS plus

39   30 micromole QCT reduced the LDH ($p = 0.1$, trend), whereas LPS plus 100 micromoles HU,

40   significantly increased LDH ($p = 0.0004$). However, LPS plus treatment with 30 micromoles

41   QCT/100 micromoles HU, significantly reduced LDH, compared with HU alone ($p = 0.0002$).

42   **Discussion**

43   These results suggest that quercetin may be effective against vascular endothelial cell damage for

44   iMAECs *in vitro*. In particular, it shows promise in preventing HU-induced cytotoxicity,

45   surprisingly found from these results. This latter finding is interesting, and should be given more

46   consideration, since HU is the only FDA-approved drug for treating sickle cell patients, and its

47   use is rapidly increasing.

48

49   **Introduction**

50

51   Sickle Cell Disease (SCD) is a genetic disease caused by a single nucleotide mutation in the beta

52   globin gene, which increases the tendency of hemoglobin to polymerize and causes red blood

53   cells (RBC) to acquire a sickle shape (Pauling & Itano, 1949). Accumulation of the polymerized

54   hemoglobin S (HbS) within the RBC causes injury to the red cell, altering both its form and

55   function. Accumulation of the damaged red blood cells within the blood vessels causes decreased

56   blood flow and hence, low oxygen delivery. The low oxygen levels promote more red cell

57   deformation, and increased polymerization in the already damaged red cells (Steinberg, 2008).

58   As these damaged cells traverse the circulatory system they encounter and injure vascular

59   endothelial cells. Hydroxyurea (HU) is the only FDA approved drug for SCD. It is a

60   chemotherapy drug approved for use at low dose in SCD patients to increase the production of

61   fetal Hb, which does not polymerize, therefore maintaining normal red cell function and life span

62   and reducing the damage caused by the sickle cells. It is important to note that the cytotoxic

63   nature of the drug, may present a problem for long term use as an SCD treatment (Brun et al.,

64   2003; Baz et al., 2012).

65

66   Vascular endothelial cells serve multiple physiological functions in normal individuals. These

67   include control of vascular tone, separating blood from the interstitial spaces and the presence of

68   molecules and receptors involved in vascular adhesion, in both health and disease (Hebbel,

69   Osarogiagbon & Kaul, 2004; Rajendran et al., 2013). Chronic inflammation in sickle cell

70   patients is invariably associated with injury to the vascular endothelium. In SCD this damage

71   leads to continuously varying activated states of vascular endothelial cells. System-wide

72   inflammation accompanies endothelial cell activation. Increased adhesion of leukocytes to the

73   vascular endothelium demonstrates evidence of this inflammatory state and is associated with

74   shortening of the white cell's life span, and acquisition of a pro-adhesive phenotype by the

75   vascular endothelial cells. Oxidative stress and reactive oxygen species also develop in the

76   vascular endothelium (Hebbel, Osarogiagbon & Kaul, 2004; Rajendran et al., 2013). The effects

77   of activation and inflammation lead to further complications within the endothelial cells, which

78   include increased levels of inflammatory cytokines and adhesion molecules, hypoxia-reperfusion

79   injury, increased reactive oxygen production and possible vaso-occlusion (Osarogiagbon et al.,

80   2000; Koo et al., 2001; Hebbel, Osarogiagbon & Kaul, 2004; Cook-Mills, Marchese & Abdala-

81   Valencia, 2011; Chirico & Pialoux, 2012).

82

83   Ischemia/reperfusion injury (IRI) occurs when the blood supply returns to tissue that was

84   previously subjected to ischemia. Reflux of oxygen and nutrients to the formerly ischemic tissue

85   destroys cellular DNA, specific proteins and the plasma membrane. The damage related to IRI

86   stems from oxidative stress, which causes a non-specific inflammatory state in the vasculature.

87   Ischemia/reperfusion injury also induces the production of oxygen free radicals. The increased

88   production of oxygen free radicals causes the formation of reactive oxygen species. The rapid

89   buildup of free radicals during the reperfusion phase of IRI causes the body's natural sources of

90   antioxidants, such as superoxide dismutase and glutathione, to be depleted during the period of

91   ischemia (Koo et al., 2001). The depletion of natural antioxidants allows oxidative damage to

92   progress unchecked causing further injury to the endothelium. A supplemental antioxidant

93   supply, such as may be obtained from the flavonoid quercetin (QCT), may help to prevent the

94   increasing free radical production (Hyacinth, Gee & Hibbert, 2010) as well as the damage to

95   macromolecules, including DNA (Potenza et al., 2008).

96

97   The literature describes quercetin as a reliable anti-oxidant for *in vitro* studies (Potenza et al.,

98   2008; Boots, Haenen & Bast, 2008; Dajas et al., 2015; D'Andrea, 2015; Sharma et al., 2015).

99   The enzyme Xanthine Oxidase is one source of reactive oxygen species production (Kelley et al.,

100 2010; Cantu-Medellin & Kelley, 2013) and reports in the literature show that QCT is an

101 effective inhibitor of xanthine oxidase (Middleton, Kandaswami & Theoharides, 2000; Pauff &

102 Hille, 2009; Cao, Pauff & Hille, 2014). The use of QCT as a supplemental source of anti-

103 oxidants may help to reduce the drain on the already weak anti-oxidant levels in SCD patients.

104 Based on the antioxidant properties of QCT, in the present study we investigated whether QCT

105   may decrease the level of cytotoxicity associated with HU treatment on an inflammation/hypoxia

106   model.

107

108   Materials and Methods

109

110   *Culture of Immortalized Mouse Aortic Endothelial Cells*

111

112   Immortalized Mouse Aortic Endothelial Cells (iMAEC), a stable cell line derived from

113   endothelium of the mouse aorta, were obtained through a generous gift from the Jo Lab at Emory

114   University (Atlanta, GA) (Ni et al., 2014). Cells were grown in Dulbecco's Modified Eagle

115   Medium (DMEM) containing 10% fetal bovine serum, 1% 100X MEM Non-Essential Amino

116   Acids Solution (MNEAA, Gibco, Waltham, MA), 1.3% Endothelial Cell Growth Supplement

117   (ECGS) (Gibco, Waltham, MA, final concentration 50µg/ml) and 1% 100X penicillin-

118   streptomycin, following previously published procedures (Koo et al., 2001). The cultures were

119   kept in a humidified chamber at 5% CO_2 and 37°C, and the medium was changed every 1 to 2

120   days.

121

122   *Treatment of Immortalized Mouse Aortic Endothelial Cells*

123

124   iMAEC were seeded at a density of 10,000 cells/well and allowed to grow to approximately 70%

125   confluency, which occurred in approximately 36 hours. After the desired confluence was

126   achieved, the iMAEC were treated with 250 ng/ml of lipopolysaccharide (Sigma-Aldrich, St.

127   Louis, MO) along with varying concentrations of Quercetin (M.W. 302.24 g/mol) (QCT)

128   (Sigma-Aldrich, St. Louis, MO), Hydroxyurea (M.W. 76.0547 g/mol) (HU) (Sigma-Aldrich, St.

129   Louis, MO) or a combination of Quercetin + Hydroxyurea (QCT+HU). The treatment with

130   QCT, HU, or QCT+HU, lasted for 24 hours. The cells were then subjected to two hours of

131   enzymatic hypoxia, which is a model for ischemia-reperfusion injury, achieved using a

132   combination glucose oxidase (GOX) (Sigma-Aldrich, St. Louis, MO) and catalase (CAT)

133   (Sigma-Aldrich, St. Louis, MO), both from Sigma-Aldrich, as previously described (Mueller,

134   Millonig & Waite, 2009). Briefly, the GOX and CAT enzymes were added to high glucose

135 DMEM (4.5 g/L glucose; Thermo-Fisher, Waltham, MA) and the resulting mixture placed on the

136 confluent iMAEC for two hours of incubation. Hypoxia was subsequently terminated by

137 removing the GOX/CAT DMEM and replacing it with normal DMEM containing 250 ng/ml of

138 LPS. The iMAEC were then allowed to incubate in the normal DMEM/LPS media for 24 hours

139 after which cytotoxicity was measured. (See Figure 1 for outline of experiment)

140

141

142 *Figure 1*

143

144 *Measurement of Cytotoxicity*

145

146 LDH released from the cells into the medium, was measured as per the manufacturer's protocol

147 (Pierce LDH Cytotoxicity Assay; ThermoFisher, Waltham, MA), and served as a biomarker for

148 cellular cytotoxicity following the cell treatments. Before starting the assay, 10μl of 10X lysis

149 buffer was added to healthy iMAEC cells and allowed to incubate at 37˚ for 45 minutes. These

150 cells served as the positive control, indicating the expected mean concentration of LDH in each

151 well. After 24 hours of normal aerobic exposure, 50μl of supernatant from each well was

152 transferred to a new 96-well plate, 50μl of substrate solution was added per the manufacturer's

153 protocol and the cells were incubated at room temperature for 30 minutes. After 30 minutes,

154 50μl of stop solution was added to each well. The absorbance for each well was then measured

155 by spectrophotometer at 490nm and 680 nm to account for background absorbance. To quantify

156 LDH release, the absorbance of each treatment group was divided by the absorbance of the

157 positive control group and multiplied by 100. Each result was expressed as a percentage of total

158 LDH release.

159

160 *Statistical Analysis*

161

162 Results are presented as means ± SD. The D'Agostino's K^2 test was used to measure the

163 distribution of the data. The data was not normally distributed; hence the Mann-Whitney U Test

164 was used to compare differences by treatment. Based on the results of the D'Agostino's K^2 test,

165 the Kruskal-Wallis non-parametric analysis of variance with pair-wise comparison was utilized.

166 Statistical significance was assumed at p≤0.05 for all analyses. No outliers, designated as SD±2,

167 were identified. The data were analyzed using GraphPad Prism 7 for MacOS Sierra.

168

169 **Results**

170

171 To establish whether LPS (Inflammatory Model, 250 ng/ml) causes increased LDH release, two

172 groups of cells were compared. The mean absorbance of the LPS treated cells was significantly

173 higher than for the untreated cells (0.60 ± 0.17 vs 0.42 ± 0.12, p=0.0004) (Figure 2). After

174 confirming that LPS causes increased LDH release, we sought to determine whether various

175 concentrations of QCT could prevent LDH release. The percentage of LDH released from the

176 30μmol QCT-treated cells trended lower than the LDH% measured in the inflammatory model

177 (LPS, 250 ng/ml) (p=0.1, trend) (Figure 3). Those cells treated with 100μmol of HU, showed a

178 significantly higher percentage of LDH release compared with LPS inflammation alone (14.4 ±

179 6.87 vs. 5.67 ± 5.73, p=0.0004). The addition of 30μmol of QCT with the HU treatment gleaned

180 a significant reduction of LDH release, compared with only HU treatment (5.55 ± 4.66 vs. 14.4 ±

181 6.87, p=0.0002). Interestingly, the combination treatment (QCT 30/100μmol HU) returned LDH

182 levels to those of the baseline LPS treated group (250 ng/ml), (Figure 4).

183

184

185

186 **Figure 2**

187 **Figure 3**

188 **Figure 4**

189

190

191 **Discussion and Conclusions**

192

193 Damage to vascular endothelial cells in SCD patients contributes considerably to the progression

194 and severity of the disease (Hebbel, Osarogiagbon & Kaul, 2004; Kaul, Finnegan & Barabino,

195 2009). One form of injury to the endothelial cells results from cyclical periods of ischemia and

196   reperfusion which exacerbate the endothelial cell damage and contribute to the progression of the

197   disease (Hebbel, 2014). Since endothelial cells are a primary site of pathogenesis, there is

198   considerable interest in developing treatments that target inflammatory mechanisms in these

199   cells. This study was designed to determine if QCT, a plant flavonoid with anti-oxidant and anti-

200   inflammatory properties, could reduce or prevent the damage caused by inflammation/hypoxia

201   and damage caused by HU. Reports in the literature indicate that LDH is both a general marker

202   of cellular damage and a marker of ischemia-reperfusion damage in SCD (Stankovic Stojanovic

203   & Lionnet, 2016). This study used LDH as a marker for assessing the efficacy of QCT treatment

204   for reducing inflammation in an in-vitro model. The first result confirmed that QCT (30μmol)

205   treatment showed a trend toward reducing the percentage of LDH released by an LPS induced

206   inflammatory model undergoing a period of hypoxia and re-oxygenation. This lends preliminary

207   support to the idea that QCT could be useful for reducing cytotoxicity caused by inflammation

208   and oxidative stress in endothelial cells. The second finding that HU (100μmol), significantly

209   increased the amount of LDH released in the inflammatory model, during a period of hypoxia

210   and re-oxygenation, is novel since to our knowledge, there are no reports in the literature about

211   this effect of low dose HU for treating SCD (Brun et al., 2003; Baz et al., 2012). By reducing the

212   damage in endothelial cells, a key site of disease progression in SCD (Osarogiagbon et al., 2000;

213   Hebbel, Osarogiagbon & Kaul, 2004; Rajendran et al., 2013; Hebbel, 2014), more severe disease

214   side effects such as atherosclerosis (Elsharawy, Moghazy & Shawarby, 2009), future clot

215   formation, and vessel stenosis (Adams, 2007; Doepp et al., 2012; Sparkenbaugh & Pawlinski,

216   2013) could be alleviated or completely prevented. However, any reduction of baseline

217   inflammation related to QCT treatment, could be reversed by subsequent inflammatory response.

218

219   While QCT may not completely avert damage caused by the combined effect of inflammation,

220   oxidative stress and HU treatment, these findings suggest that the QCT may prevent injury from

221   the HU drug, shown in these studies to cause inflammation, but which has previously been

222   assumed only to reduce the subclinical ischemic events induced by sickle red blood cells. The

223   combination of QCT and HU, resulted in significant reduction of LDH release to baseline levels

224   of inflammation, indicating that QCT may completely prevent damage induced by HU treatment,

225   possibly returning the endothelial cells to pre-treatment levels. Therefore, a combination

226   treatment of QCT with the HU, may prevent or reduce HU treatment induced endothelial cell

227   damage. Suggested QCT use as adjunct to HU therapy may translate to improved disease

228   outcomes.

229

230   This study was limited to investigating only one type of endothelial cell, aortic endothelial cells.

231   While there is vast homogeneity among endothelial cell types there are still subtle differences

232   that may affect the outcome of QCT treatment (Aird, 2012). It is a vital future step to repeat

233   these experiments in endothelial cells from several different locations in the body and of human

234   origin. This could confirm QCT efficacy system wide. Once confirmed *in vitro* then in vivo

235   models will need to be tested.

236

237   In summary, this study demonstrates a new and important finding that HU treatment could be

238   increasing inflammation in the already chronically inflamed vascular endothelium in patients

239   with SCD. The study successfully demonstrated that QCT may be an effective treatment in

240   reducing cytotoxicity caused by inflammation resulting from ischemia-reperfusion, and by

241   treatment with HU. Given that HU is an important pharmacological agent used in the treatment

242   of SCD, it may advantageous to reduce possible short and long term cytotoxic effects associated

243   with HU treatment. These studies show that pre-treatment with QCT may provide protection

244   from endothelial cell damage caused by inflammation and hypoxic periods. Use of QCT as an

245   adjunct treatment with HU could reduce the amount of HU required, limit endothelial cell injury

246   and possibly decrease the severity of secondary disease consequences, such as stroke, vessel

247   stenosis, and vasculopathy. Based on the results of this study, adding QCT to the standard

248   treatment regimen should be seriously considered.

249

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258

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350

351

Figure 1

Experimental Outline

Timeline of Experimental Procedure

Measure Cytotoxicity

24 hours	2 hours	24 Hours
Normoxia	Hypoxia	Normoxia

No treatment, LPS, LPS+QCT,
LPS+HU+QCT

Media
Change

Media
Change
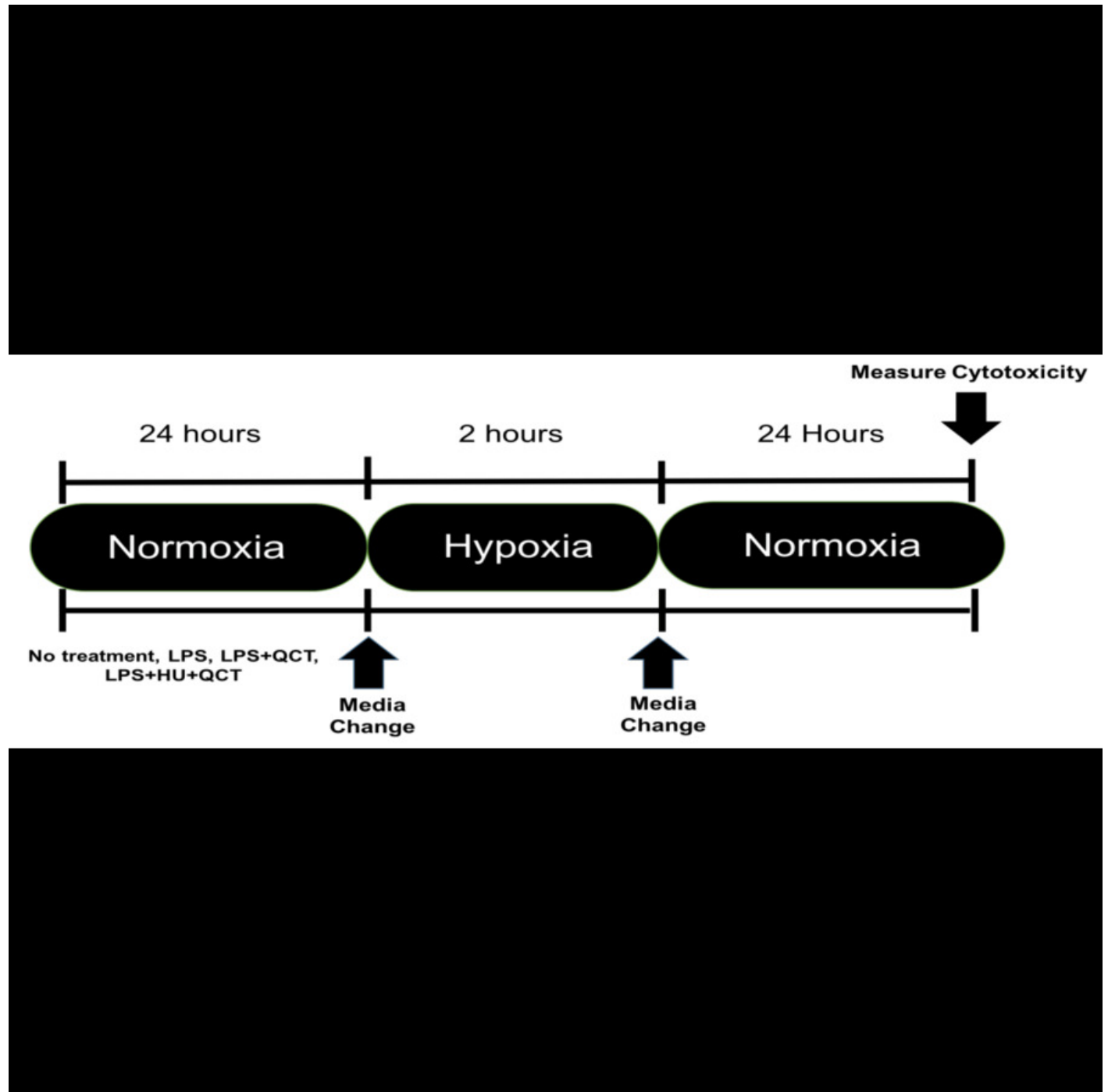
Figure 2

Comparing LDH release in LPS treated cells versus non-treated cells.

Values are means +/- SD. The mean LDH release of the LPS treated cells was significantly higher than for the untreated cells.
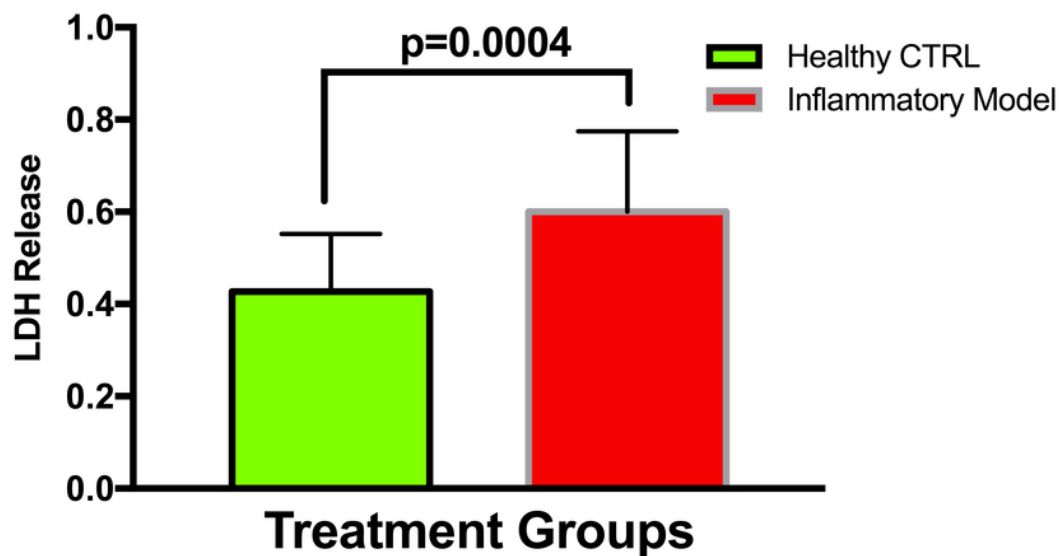
Figure 3

The effect of various concentrations of quer on LDH release.

Values are means +/- SD. The percentage of LDH released from the 30μmol QCT-treated cells trended lower than the LDH release measured in the inflammatory model.
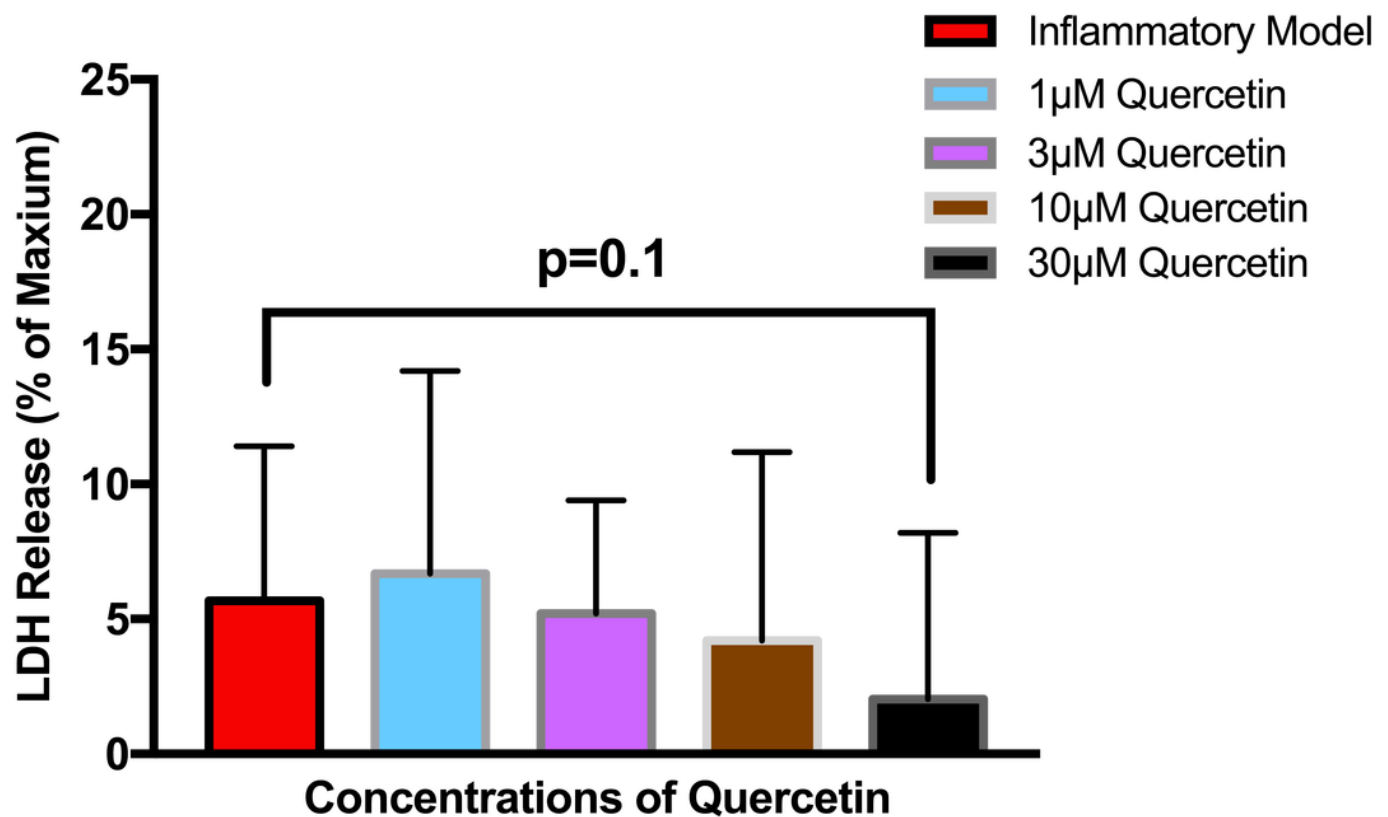
Figure 4

The effects of hydroxyurea and quercetin on LDH release.

Values are means +/- SD. The addition of 30μmol of QCT in combination with the HU treatment gleaned a significant reduction of LDH release, compared with only HU treatment.