1 **Metformin attenuates myocardium dicarbonyl stress induced by chronic**
2 **hypertriglyceridemia.**
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Abstract

Aim Reactive dicarbonyls stimulate production of advanced glycation endproducts, increase oxidative stress and inflammation and contribute to the development of vascular complications. We measured concentrations of dicarbonyls - methylglyoxal (MG), glyoxal (GL) and 3-deoxyglucosone (3-DG) - in the heart and kidney of a model of metabolic syndrome - hereditary hypertriglyceridemic rats (HHTg) and explored its modulation by metformin.

Methods Adult HHTg rats were fed a standard diet with or without metformin (300mg/kg b.wt.) and dicarbonyl levels and metabolic parameters were measured.

Results HHTg rats had markedly elevated serum levels of triacylglycerols ($p<0.001$), FFA ($p<0.01$) and hepatic triacylglycerols ($p<0.001$) along with increased concentrations of reactive dicarbonyls in myocardium (MG: $p<0.001$; GL: $p<0.01$; 3-DG: $p<0.01$) and kidney cortex (MG: $p<0.01$). Metformin treatment significantly reduced reactive dicarbonyls in the myocardium (MG: $p<0.05$, GL: $p<0.05$, 3-DG: $p<0.01$) along with increase of myocardial concentrations of reduced glutathione ($p<0.01$) and glyoxalase 1 mRNA expression ($p<0.05$). Metformin did not have any significant effect on dicarbonyls, glutathione or on glyoxalase 1 expression in kidney cortex.

Conclusion Chronically elevated hypertriglyceridemia was associated with increased levels of dicarbonyls in heart and kidney. Beneficial effects of metformin on reactive dicarbonyls and glyoxalase in the heart could contribute to its cardioprotective effects.

Keywords: hypertriglyceridemia, dicarbonyl stress, methylglyoxal, glyoxalase, metabolic syndrome, metformin

60 **Abbreviations** AGE – advanced glycation end product, CML – carboxymethyl lysine, FFA –
61 free fatty acids, GSH – reduced form of glutathione, GSSG – oxidized form of glutathione,
62 TBARS – thiobarbituric acid reactive substance, TAG – triacylglycerol, MG – methylglyoxal,
63 GL- glyoxal, 3-DG 3-deoxyglucosone, Glo-1 – glyoxalase 1
64
65
66 **Introduction**
67
68 The protein glycation caused by reactive dicarbonyls stimulates the production of advanced
69 glycation end products (AGEs) and subsequently contributes to the development of chronic
70 vascular complications, in particular in patients with diabetes (Schalkwijk *et al* 2015). Under
71 normal conditions, the excessive protein glycation is prevented through glutathione-dependent
72 glyoxalase detoxification. An impaired balance between the generation of dicarbonyls and the
73 efficiency of their scavenger pathways leads to dicarbonyl stress (Rabbani *et al* 2015). Both of
74 these processes are impaired in diabetic patients, where dicarbonyl generation is increased and
75 glyoxalase activity including glutathione status is decreased (Maessen *et al* 2015). Dicarbonyl
76 stress is involved in the pathogenesis of metabolic syndrome, as well as in diabetic macro-
77 and microvascular complications. Higher plasma levels of methylglyoxal are observed in type
78 1 and 2 diabetic patients (Fleming *et al* 2012) and in obese patients with metabolic syndrome
79 (Uribarri *et al* 2015). In addition, it has been reported that methylglyoxal administration
80 induces endothelial dysfunction, oxidative stress and impaired vasodilatation (Sena *et al*
81 2012), and increases macrophage infiltration in adipose tissue in experimental studies
82 (Matafome *et al* 2012). An excessive generation of dicarbonyl species such as methylglyoxal
83 (MG) is typically associated with hyperglycemia and high glucose variability (Maessen *et al*

84 2015), nevertheless its other possible inductors include also dyslipidemia and insulin
85 resistance. (Tenenbaum *et al* 2014)
86 Metformin, the most widely prescribed glucose-lowering agent for the treatment of type 2
87 diabetes, has been proposed as a scavenger of reactive dicarbonyl species. It has been
88 previously demonstrated that metformin, through the guanidine group, can bind to
89 methylglyoxal (Kinsky *et al* 2016), and that metformin treatment is able to reduce plasma
90 methylglyoxal levels in patients with type 2 diabetes (Kender *et al* 2016). We have previously
91 demonstrated in a rat model of chronic inflammation that metformin administration decreased
92 methylglyoxal levels in heart (Malinska *et al* 2016).
93 In the current study we measured concentrations of dicarbonyls in the heart and the kidney of
94 a rodent model of metabolic syndrome - non-obese hereditary hypertriglyceridemic rats. This
95 strain originating from Wistar rats is characterized by severe hypertriglyceridemia, insulin
96 resistance, hyperinsulinemia, hepatic steatosis and oxidative stress with an absence of obesity
97 and hyperglycemia thus representing an experimental model of metabolic syndrome (Kazdova
98 *et al* 1997, Zicha *et al* 2006). We hypothesized that severe hypertriglyceridemia and insulin
99 resistance will be associated with increased dicarbonyl levels even in the absence of
100 hyperglycemia and that metformin treatment will reduce dicarbonyls in both the heart and the
101 kidney.
102
103 **Methods**
104 *Animals and diet*
105 All experiments were performed in agreement with the Animal Protection Law of the Czech
106 Republic (311/1997) and were approved by the Ethics Committee of the Institute for Clinical
107 and Experimental Medicine.

108 Six-month old Wistar male rats obtained from Charles River Laboratories (controls) and the
109 non-obese hereditary hypertriglyceridemic strain of rats (HHTg) were used in this study. The
110 rats were fed a standard laboratory diet with or without metformin at a dose of 300 mg/kg b.
111 wt. for 4 weeks. At the end of experiments, animals were sacrificed in a postprandial state.
112
113 *Analytic methods/ Biochemical analyses*
114 Serum levels of triacylglycerols, glucose, total cholesterol, HDL-cholesterol and FFA were
115 measured using commercially available kits (Erba Lachema, Czech Republic and Roche
116 Diagnostics, Germany). Serum insulin and carboxymethyl lysine (CML) concentrations were
117 determined using a Mercodia Rat Insulin ELISA kit (Mercodia AB, Sweden) and a Rat CML
118 ELISA kit (Mybiosource, USA). Plasma and urine lactate were analyzed electrochemically
119 using ion-selective electrodes (Radiometer, Czech Republic).β-Hydroxybutyrate and
120 acetoacetate plasma concentrations were determined using an enzymatic method, as
121 previously described (Galán *et al* 2001).
122 For the oral glucose tolerance test (OGTT), blood glucose was determined after a glucose load
123 (3g of glucose/kg b.wt.) administered intragastrically after overnight fasting. The blood
124 glucose concentration were determinated through analysis of blood samples collected from
125 the tail at 0, 30, 60, 120 min after glucose loading. The area under curve (AUC) for glucose
126 was calculated over the 120 min period.
127 For determination of tissue triacylglycerols, samples were extracted in chloroform/methanol
128 and futher processed as described previously (Malinska *et al* 2015).
129 Levels of reduced (GSH) and oxidized (GSSG) forms of glutathione were determined using a
130 high-performance liquid chromatography method with fluorescent detection in accordance
131 with the HPLC diagnostic kit (Chromsystems, Germany).
132

133 ***Dicarbonyl stress parameters***: Dicarbonyl concentrations were determined after
134 derivatization with 1,2-diamino-benzene and using the HPLC method with fluorescence
135 detection according to Fleming and Bierhaus (Thornalley *et al* 1999).
136 Glo-1 activity was analyzed using the method described by Arai (Arai *et al* 2014). Red blood
137 cells were collected by centrifugation of blood (EDTA) samples and washed 3 times with 0.01
138 M PBS (pH 7.4). Washed cells were lysed using cold deionized water. Hemoglobin
139 concentrations were determined according to the Drabkin´s assay (Sigma).
140
141 ***Glyoxalase 1 mRNA expression:***
142 Total RNA was isolated from the kidney cortex and left ventricle using RNA Blue (Top-bio,
143 Czech Republic). Reverse transcription and quantitative real-time PCR analyses were
144 performed using the TaqMan RNA-to C_T 1-Step Kit and TaqMan Gene Expression Assay
145 (Applied Biosystems, USA) and carried out using a ViiA™ 7 Real Time PCR System
146 (Applied Biosystems, USA). Relative expression of *Glo-1* was determined after normalization
147 against *β-actin* as an internal reference and calculated using the $2^{-\Delta\Delta Ct}$ method.
148
149 ***Cell cultures, treatment***
150 Confluent Human Kidney HEK293 cells were cultivated in a control medium (DMEM,
151 Hyclone, USA supplemented with 10% FBS, Biochem, Germany) and treated with either
152 0,5mM metformin or a combination of 0,5mM metformin and 10mM lactate (Sigma) for 18 h.
153 Cells were then trypsinized and methylglyoxal content was determined in aliquots containing
154 $15*10^6$ cells according to the method described above.
155
156 ***Statistical analysis***

157 Statistical analysis was performed using either a one-way ANOVA Kruskal-Wallis test with
158 multiple comparisons or a Mann Whitney test. A value of $p<0.05$ was considered to be
159 statistically significant. The Pearson correlation was calculated to determine the relationship
160 between glutathione and methylglyoxal in the myocardium. Data are presented as mean ±
161 SEM with 95% CI.
162
163 **Results**
164
165 *The effect of hypertriglyceridemia on basal metabolic parameters*
166 Compared with controls, hypertriglyceridemic rats exhibited markedly elevated serum levels
167 of triacylglycerols, FFA and ectopic triacylglycerol accumulation in the liver and muscle,
168 impaired glucose tolerance, hyperinsulinemia and increased AGE product carboxymethyl
169 lysin (CML) and keton bodies (**Table 1**).
170 In hypertriglyceridemic rats we observed markedly increased serum levels of methylglyoxal
171 (1.802±0.121 vs 0.662±0.161 nmol/ml, $p<0.01$). Concentrations of individual reactive
172 dicarbonyls in the myocardium and kidney cortex were significantly elevated in HHTg rats
173 (**Figure 1**) compared to normotriglyceridemic controls.
174 Hypertriglyceridemia was also associated with impaired glutathione metabolism in the
175 myocardium as shown in **Figure 2a**. The reduced form of glutathione was decreased and the
176 oxidized form of glutathione was increased in the myocardium of HHTg rats.
177
178 *The effect of metformin*
179 Metformin administration to HHTg rats mildly reduced body weight and had a positive effect
180 particularly on lipid metabolism compared to untreated HHTg rats (**Table 1**).

181 As regards carbonyl stress, metformin treatment significantly reduced serum levels of
182 methylglyoxal (0.915±0.219 vs 1.802±0.121 nmol/ml, $p<0.01$), but other dicarbonyls in the
183 serum did not change. As shown in **Figure 1**, metformin treatment was associated with
184 significantly reduced levels of all measured dicarbonyls in the myocardia of HHTg rats.
185 However, there was no significant effect of metformin on dicarbonyl concentrations in the
186 kidney cortex (**Figure 1**).
187 Concentrations of hydroxybutyrate, lactate and acetoacetate in plasma and urine were
188 significantly elevated in metformin-treated HHTg rats compared to untreated rats (**Figure 4**).
189 Incubation with metformin significantly reduced the concentration of MG in the human
190 kidney HEK293 cell culture. However, the presence of lactate in the medium reduced the
191 effect of metformin on MG in isolated kidney cells (**Figure 4**).
192
193 *The effect of metformin on glutathione*
194 In the myocardium we observed improved glutathione metabolism in HHTg metformin-
195 treated rats (**Figure 2**), an elevation in the reduced form of glutathione and a decrease in the
196 oxidized form of glutathione. This effect of metformin on glutathione was not observed in the
197 kidney cortex (**Figure 2**). A direct relationship between methylglyoxal and reduced
198 glutathione in the myocardium was confirmed by negative correlation (**Figure 2c**).
199
200 *The effect of metformin on glyoxalase 1 expression and activity*
201 Gene expression of mRNA Glo-1 was increased in the myocardium (left ventricle) after
202 metformin treatment, whereas mRNA Glo-1 expression in the kidney cortex did not differ
203 between metformin-treated and -untreated HHTg rats (**Figure 3**). Metformin administration
204 also significantly increased glyoxalase 1 activity measured in red blood cells compared to
205 untreated rats.

206

207

208 **Discussion**

209

210 One of the unifying hypotheses connecting diabetes with its chronic complications suggests
211 that enhanced metabolic flux and the deleterious effects of high glucose levels are mediated
212 by the generation of toxic metabolites (Fleming *et al* 2012). Of these, reactive dicarbonyls are
213 among the most important (Rabbani *et al* 2015). Interestingly, increased dicarbonyls
214 production has also been described in patients with metabolic syndrome and dyslipidemia
215 without overt diabetes suggesting their possible involvement in the incraesed risk in
216 cardiovascular complications in these patients (Rabanni *et al* 2016). The results of our study
217 demonstrate for the first time that chronicaly elevated triglyceride and FFA levels, in the
218 absence of obesity are associated with increased production of reactive dicarbonyl species, in
219 particular methylglyoxal. In addition to its increased circulating levels, we observed also
220 markedly elevated tissue levels of dicarbonyls. Previous studies have shown that MG and GL
221 can be produced from oxidized lipids, both within their degradation and during
222 lipoperoxidation (Turk *et al* 2011) or by increased glyceroneogenesis in triacylglycerols/FFA
223 cycle (Masania *et al* 2016). Although lipid metabolism in myocardium and kidney is slightly
224 different, the elevation of dicarbonyls in these tissues in HHTg rats is nearly the same so is
225 implausible to significantly influence the creation of dicarbonyls. Other possible mechanisms
226 of hypertriglyceridemia-induced dicarbonyl accumulation include increased oxidative stress,
227 increased ketogenesis and subsequent AGE formation (Dornadula *et al* 2015). Our
228 experimental results in hypertriglyceridemic rats support the increasing evidence that
229 chronically increased lipids can be as important as carbohydrates in the stimulation of
230 excessive reactive dicarbonyl species production.

231 The massive accumulation of dicarbonyls in the myocardium of hypertriglyceridemic rats in
232 our study was associated with an impaired balance of GSH status. It has been shown that
233 adequate levels of the reduced form of glutathione are important for optimal activity of the
234 glyoxalase system, which is involved in the detoxification of MG and GL (Rabanni *et al*
235 2016). An inverse relationship between MG and reduced glutathione in the myocardium
236 suggests a possible direct relationship. One the mechanisms could be a MG-induced
237 deactivation of the antioxidant enzyme glutathione reductase thus further enhancing the
238 potential for oxidative stress damage. Other studies have shown that high serum and adipose
239 tissue levels of MG are closely related to insulin resistance in fructose-fed rats (Jia *et al*
240 2007), and MG treatment *in vitro* impairs insulin-signaling activation in skeletal muscle cells
241 (Riboulet-Chavey *et al* 2006) through increased oxidative stress and direct effects on insulin
242 signalling pathway (Nigro *et al* 2014).
243 In our current study, we focused on the effects of metformin treatment on dicarbonyl levels
244 and its metabolic consequences. Previous studies have shown that metformin may have
245 numerous beneficial effects independent of its glucose lowering properties including
246 cardioprotective effects (Rena *et al* 2013). Our previous study in SHR rats with transgenic
247 expression of human CRP (Malinska *et al* 2016) demonstrated metformin-induced decrease of
248 methylglyoxal in the heart. Here we focused on the possible mechanisms that could explain
249 metformin effects on dicarbonyl stress. In our current study in hypertriglyceridemic rats,
250 metformin treatment reduced dicarbonyl accumulation and increased Glo-1 expression in the
251 myocardium. Both of these changes could have contributed to and partly explain the
252 cardioprotective effects of metformin seen in clinical practice. Other studies have shown that
253 metformin improves the GSH/GSSG balance in the myocardium and prevents dicarbonyl
254 accumulation as a cofactor of the glyoxalase system (Ashour *et al* 2012, Foretz *et al* 2014).

255 Metformin has also been proposed as a scavenger of methylglyoxal (Rena *et al* 2013, Kinsky
256 *et al* 2016).
257 Our data show that metformin can decrease MG directly through the activation of its key
258 detoxification enzyme, Glo-1. Another important mechanism involves the interaction and
259 activation of redox-sensitive transcription factors such as Nrf2, AP1 and NFkB which can
260 again upregulate Glo-1 transcription (Xue *et al* 2012). At the transcriptional level, apart from
261 Glo-1, metformin has been also shown to restore key antioxidant defense enzymes such as
262 glutathione-S-transferase and catalase (Kender *et al* 2014).
263 In our study, untreated HHTg rats had elevated circulating levels of ketone bodies which were
264 further increased by metformin treatment. Metformin is capable to readdress fatty acid
265 metabolism from lipogenesis towards fat oxidation and ketone body production, so increased
266 β-hydroxybutyrate after metformin administration can associated with increased fatty acid
267 oxidation. Although the development of severe lactate acidosis is perceived as a negative
268 consequence associated with metformin administration (DeFronzo *et al* 2016) recent trials
269 with novel antidiabetic drugs gliflozins have suggested that moderate ketone bodies elevation
270 could have the potencial to improve myocardium metabolism (Ferrannini *et al* 2016). Recent
271 studies have reported that the failing heart relies on keton bodies as a significant alternative
272 fuel, when the fatty acids utilization is diminished (Aubert *et al* 2016). Accumulation of
273 ketone bodies in the myocardium occurs as a compensatory response against oxidative stress
274 (Nagao *et al* 2016). It is thus tempting to speculate that increased ketone bodies seen in our
275 study can also generally contribute to cardioprotective effect of metformin.
276 Interestingly, while we observed a significant metformin-induced attenuation of dicarbonyl
277 stress in the heart no such effects could be seen in the kidney. In our study, an incubation of
278 isolated human kidney cell cultures with metformin rapidly reduced MG concentrations, but
279 this effect was abolished in the presence of lactate. Likewise, the presence of lactate reduced

280 the effect of metformin on dicarbonyl stress in kidney cells. Taken together our data suggest
281 that the lack of improvement of dicarbonyl stress in the kidney as compared to myocardium
282 could be due to high levels of lactate in the kidney that abolish metformin effects.
283 In summary, our results indicate that chronically elevated hypertriglyceridemia and FFA are
284 associated with increased levels of methylglyoxal in serum and with markedly elevated
285 reactive carbonyls in the heart and kidney. The beneficial effect of metformin administration
286 on reactive dicarbonyls and glyoxalase 1 in the heart could contribute to the cardioprotective
287 effect of metformin independently of its antihyperglycemic effect. It remains to be shown
288 whether similar organ-specific effects of metformin on dicarbonyl stress can also be detected
289 in humans.
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291
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298 **Duality of interest**
299 The authors declare that there is no duality of interest associated with this manuscript.
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302 **References**
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TABLE 1: **The effects of hypertriglyceridemia and metformin on metabolic parameters.**

	Wistar	HHTg	P1 <	HHTg + metformin	P2 <
Body weight g	480 ± 22	483 ± 23	NS	450 ± 12	0.05
Serum triglycerides mmol/l	1.37 ± 0.23	4.78 ± 0.43	0.01	2.39 ± 0.13	0.02
FFA mmol/l	0.19 ± 0.03	0.83 ± 0.06	0.01	0.70 ± 0.08	0.05
Cholesterol mmol/l	1.72 ± 0.10	1.54 ± 0.10	NS	1.91 ± 0.33	NS
HDL-C mmol/l	1.24 ± 0.05	0.75 ± 0.03	0.01	1.23 ± 0.08	0.02
Triglycerides in the liver μmol/g	4.32 ± 0.7	13.87 ± 2.23	0.01	9.20 ± 1.22	0.05
Triglycerides in muscle μmol/g	4.96 ± 1.95	8.43 ± 1.64	0.05	8.55 ± 1.70	NS
Fasting glucose mmol/l	3.86 ± 0.13	5.30 ± 0.27	0.05	4.49 ± 0.26	NS
Insulin pmol/l	469 ± 30	580 ± 83	0.05	225 ± 28	0.01
AUC_{0-120} mmol/l	674 ± 9	787 ± 19	0.05	818 ± 44	NS
β-hydroxybutyrate μmol/l	45.5 ± 2.6	91.6 ± 2.9	0.01	127.9 ± 6.3	0.01
Acetoacetate μmol/l	27.1 ± 4.9	44.3 ± 6.6	0.01	39.1 ± 4.9	NS
CML ng/ml	104.7 ± 1.0	131.0 ± 6.5	0.05	130.8 ± 1.1	NS
GSH/GSSG in myocardium	4.01 ± 0.16	2.15 ± 0.09	0.05	4.65 ± 0.26	0.01
GSH/GSSG in kidney cortex	20.22 ± 0.87	20.04 ± 0.26	NS	18.48 ± 0.13	NS

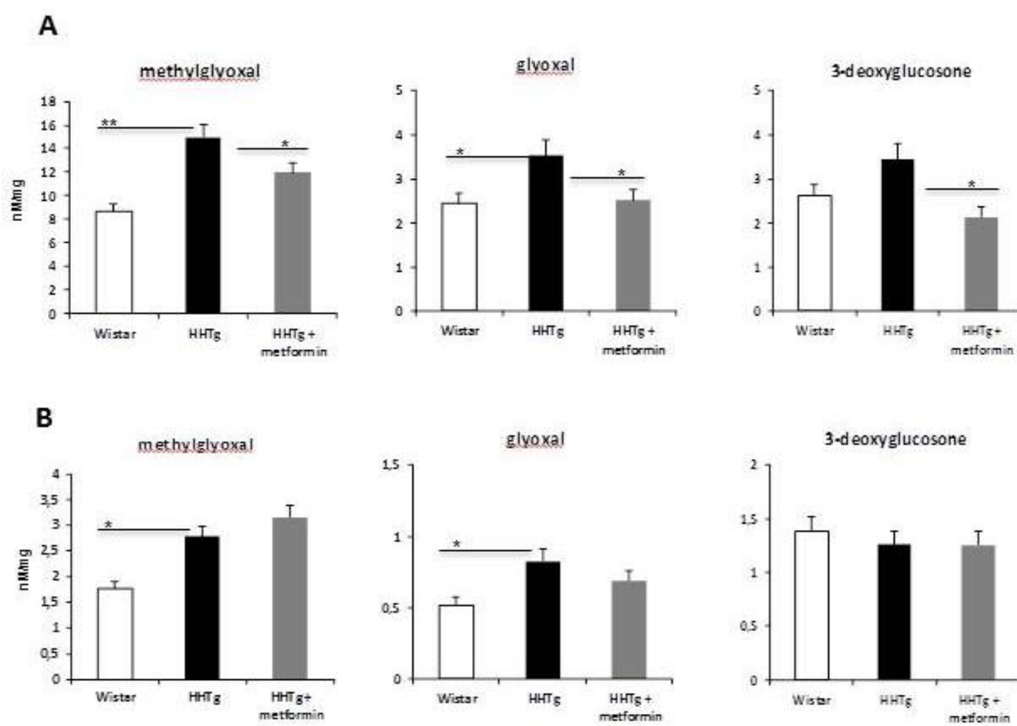
Data are mean ± SEM. n=8
P1 – HHTg vs Wistar
P2 – HHTg + metformin vs HHTg

Figure legends:

Figure 1:

The effects of hypertriglyceridemia and metformin on dicarbonyl levels in myocardium (A) and kidney cortex (B).

Data are expressed as mean ±SEM. *denote p<0.05, ** denote p<0.01



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466 **Figure 2**:

467 **The effects of hypertriglyceridemia and metformin on glutathione in myocardium (A)**

468 **and kidney cortex (B) and the relationship between methylglyoxal and glutathione in**

469 **myocardium (C), Spearman's correlation coefficient R^2=0.5882, p<0.05.**

470 Data are expressed as mean ± SEM. *denote p<0.05, ** denote p<0.01

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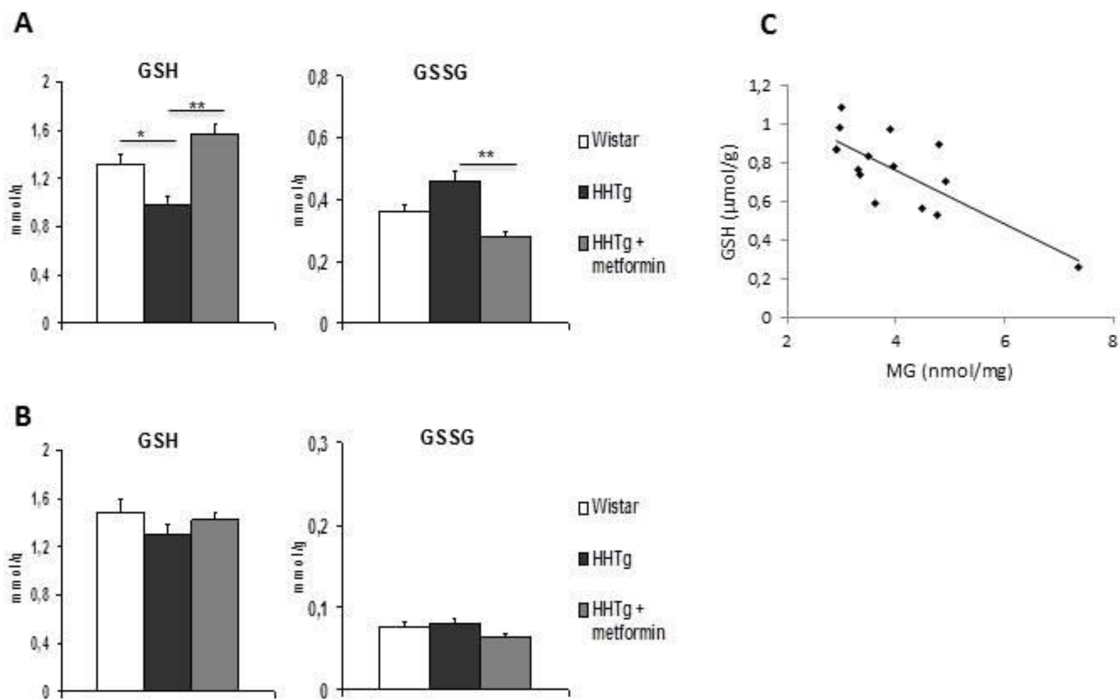

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484 **Figure 3**:

485 **The effect of metformin on glyoxalase 1 mRNA expression in myocardium (A) and**

486 **kidney cortex (B) and on glyoxalase 1 activity in erytrocytes (C).**

487 Values are presented as mean ± SEM. ∗ denote $p<0.05$ compared to HHTg.

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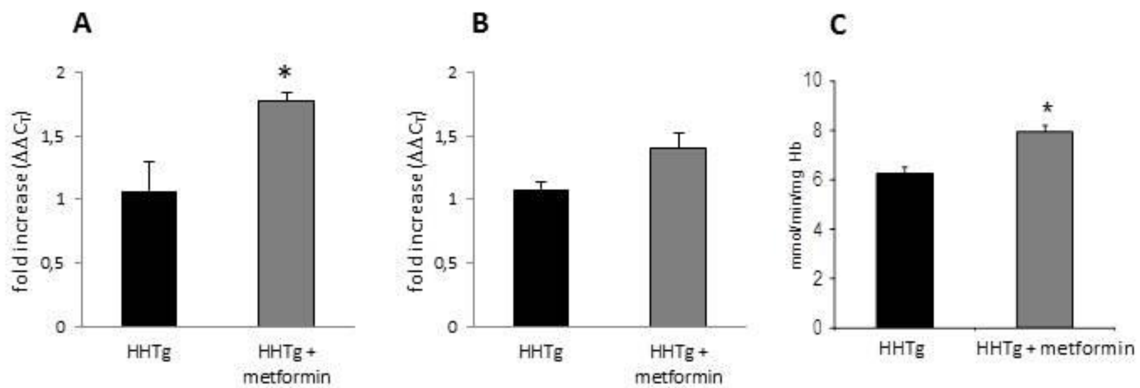

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493 **Figure 4**:

494 **The effect of metformin on lactate in plasma (A) and urine (B) and *in vitro* on human**

495 **kidney cells (C).** (C – control, M – metformin, M+L – metformin + lactate)

496 Data are expressed as mean ± SEM. *denote $p<0.05$

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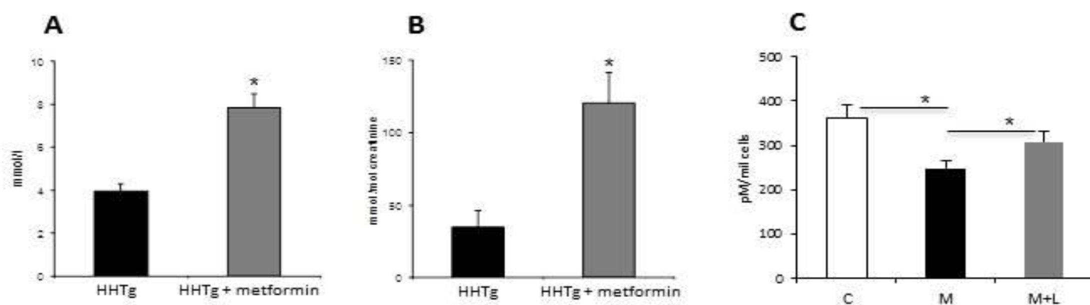

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