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Title: Inhibition of Kupffer cell functions modulates arsenic intoxication in Wistar rats
Running title: Kupffer cells mediate arsenic toxicity
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Abstract
Study determined the influence of the inhibition of Kupffer cell functions by GdCl3 in arsenic intoxication. Twenty-four Wistar rats weighing between 150 and 160 g were randomly assigned into four groups. Group 1 received sodium arsenite (1.5 mg/kg body weight) once a day, group 2 received GdCl3 (2 mg/kg body weight) once, 24 hours before commencing the arsenite (1.5 mg/kg body weight) treatment. Group 3 received GdCl3 (2 mg/kg body weight) once and subsequently given distilled water. Group 4 received distilled water only. The treatments were daily by oral gavage and lasted for 28 days. Animals were euthanized 24 hours after the last treatment. Arsenic exposure elevated the activities of rat plasma AST, ALT, ALP and γ-GT, indicative of liver injury. Arsenic exposure in rat lowered GSH concentration but potentiated inflammation and oxidative stress evidenced in the raised levels of MPO, NO and MDA. Rats with arsenic exposure were predisposed to atherosclerosis, lowering the HDL-C but elevated the LDL-C concentration. The histopathological assessment showed degenerating cellular lesion caused by arsenic. However, the inhibition of Kupffer cell functions by GdCl3 suppressed arsenic intoxication; improving the liver function indices, oxidative stress status, lipid profile, neutrophilic inflammation and ultimately restored the cellular architecture. Data suggest that specific inhibition of Kupffer cells by GdCl3 protected against arsenic intoxication.

Keywords: Arsenic; Atherosclerosis; Hepatotoxicity; Kupffer cells; Metals

Changelog
1. Reviewers' comments:
• Reviewer #1:
the author added "Further, studies have shown that specific
• 72 inhibition of Kupffer cells by GdCl3 offered some beneficial effects against development
• 73 of hepatic injury (Ding et al., 2003; Jorgensen et al., 2001)." in the introduction, which gives the references to indicating the specificity of GdCl3. However, a brief summary on how they prove that GdCl3 should be included.

Response: The section has been modified accordingly to accommodate the needed details as pointed out by the reviewer.

2. Reviewer #2:
Adeyemi studied the mechanism of the protective effect of $GdCl_3$ on arsenic intoxication. They found inhibition of Kupffer cell function by $GdCl_3$ protected against arsenic intoxication and improved liver function. Although the quality of the manuscript improved a lot, however, there are still some issues need to be clarified.

- The results should be present in the form of several subheadings. And the authors claimed the oral exposure of rats to arsenic for 28 days significantly elevated the liver function. This description is not corrected. The increased plasma AST, ALT, ALP indicated that the liver function was damaged.

Response: The affected portion has been corrected accordingly.

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Inhibition of Kupffer cell functions modulates arsenic intoxication in Wistar rats

***Running title**: Kupffer cells mediate arsenic toxicity*

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ABBREVIATIONS:

$GdCl_3$ – gadolinium chloride; MDA – malondialdehyde; GSH – reduced glutathione; MPO – myeloperoxidase; NO – nitric oxide; AST – aspartate transaminase; ALT – alanine transaminase; ALP – alkaline phosphatase; γ-GT – γ glutamyl transferase; HDL-C – high density lipoprotein cholesterol; LDL-C – low density lipoprotein cholesterol; CHOL – total cholesterol; AI – atherogenic index

22 **Abstract**

23 Study determined the influence of the inhibition of Kupffer cell functions by $GdCl_3$ in
24 arsenic intoxication. Twenty-four Wistar rats weighing between 150 and 160 g were
25 randomly assigned into four groups. Group 1 received sodium arsenite (1.5 mg/kg body
26 weight) once a day, group 2 received $GdCl_3$ (2 mg/kg body weight) once, 24 hours
27 before commencing the arsenite (1.5 mg/kg body weight) treatment. Group 3 received
28 $GdCl_3$ (2 mg/kg body weight) once and subsequently given distilled water. Group 4
29 received distilled water only. The treatments were daily by oral gavage and lasted for 28
30 days. Animals were euthanized 24 hours after the last treatment. Arsenic exposure
31 elevated the activities of rat plasma AST, ALT, ALP and γ-GT, indicative of liver injury.
32 Arsenic exposure in rat lowered GSH concentration but potentiated inflammation and
33 oxidative stress evidenced in the raised levels of MPO, NO and MDA. Rats with arsenic
34 exposure were predisposed to atherosclerosis, lowering the HDL-C but elevated the
35 LDL-C concentration. The histopathological assessment showed degenerating cellular
36 lesion caused by arsenic. However, the inhibition of Kupffer cell functions by $GdCl_3$
37 suppressed arsenic intoxication; improving the liver function indices, oxidative stress
38 status, lipid profile, neutrophilic inflammation and ultimately restored the cellular
39 architecture. Data suggest that specific inhibition of Kupffer cells by $GdCl_3$ protected
40 against arsenic intoxication.

41 **Keywords**: Arsenic; Atherosclerosis; Hepatotoxicity; Kupffer cells; Metals

42

43 **Introduction**

44 Arsenic, as trivalent (As^{3+}) and pentavalent salt (As^{5+}), is released into the environment
45 via agricultural, industrial and medical applications (Aliyu et al., 2012). Arsenic is a toxic
46 metalloid and a common contaminant of drinking water. Arsenical exposure through
47 drinking water is common and occurs in many areas of the world (Morakinyo et al.,
48 2010). Consumption of arsenic-contaminated water increases risk of cardiovascular
49 disease, lung disease, hepatic disease, and cancer in millions of people worldwide (Das
50 et la., 2012; Kumar et al., 2014; Okoji et al., 2002; Straub et al., 2008). Arsenic-
51 contaminated drinking water is associated with several adverse health effects in many
52 developing areas around the world, including China, India, Mexico, and Bangladesh. [2, 3]

53

54 Arsenite intoxication limits ATP production (Hughes et al., 2011), while potentiating
55 oxidative stress which readily leads to cellular dysfunction (Rana et al., 2011). While the
56 mechanism of arsenic toxicity remains limited, current treatment and preventive
57 measures for arsenic intoxication are still elusive (Chowdhury et al., 2006). Therefore,
58 identifying the cellular factors that contribute to modulate the liver response to
59 arsenicals is central to understanding their toxicology. We reasoned that, identifying
60 cellular factors involved in liver response to arsenic intoxication would further aid our
61 understanding of its mode of toxic action as well as provide opportunity for the
62 development of newer treatment strategy.

63

64 Kupffer cells are macrophage native to the liver and mediate hepatic response to
65 chemical assault (Roberts et al., 2007). Generally, Kupffer cells play an important role in
66 the normal physiology and homeostasis of the liver as well as participate in the acute

67 and chronic responses of the liver to toxic compounds and have been shown to
68 modulate hepatic injury (Roberts et al., 2007; Yee et al., 2000). Several studies have
69 demonstrated the central role played by Kupffer cells in liver responses to the toxic
70 action of several drugs (Ito et al., 2003; Ju et al., 2002; Kresse et al., 2005; Prins et al.,
71 2004; Yao et al., 2004; Yee et al., 2000). Meanwhile, it has been demonstrated that
72 pretreatment of mice with $GdCl_3$, a specific inhibitor of Kupffer cells offered protection
73 against liver injury (Jorgensen et al., 2001). Further, in a separate investigation, Ding et
74 al (2003) showed that the intraperitoneal administration of $GdCl_3$ to mice at doses
75 between 10 – 20 mg/kg caused apoptosis of Kupffer cells and blocked the Kupffer cell
76 effector function without necessarily damaging the liver. Currently, there is no empirical
77 data on how Kupffer cells affect arsenic toxicity. Therefore, determining the role Kupffer
78 cells play in arsenical intoxication may be essential to understanding the mode of the
79 toxic action. To this end, this study evaluated the influence of the inhibition of Kupffer
80 cell functions by $GdCl_3$ in experimental arsenic intoxication.

81

82

83 **Materials and methods**

84 **Chemicals reagents**

85 All chemicals and reagents used were of analytical grade. Sodium arsenite ($NaAsO_2$)
86 and $GdCl_3$ were products of Sigma Chemicals (St. Louis, MO, USA). Reagent assay kits
87 for aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-Glutamyl
88 transferase (γ-GT), total cholesterol (CHOL), high density lipoprotein (HDL), bilirubin,
89 and albumin were products of Randox Laboratories, (Crumlin, UK).

90 **Experimental animals**

91 Twenty-four male Wistar rats weighing between 150 and 160 g were purchased from
92 the experimental animal farm at the Landmark University, Nigeria. The rats were
93 housed in plastic cages and kept in a well-ventilated room, with alternate light and dark
94 cycle (12 hours each). The animals were acclimatized for 14 days before the treatment
95 commenced. The animals were fed standard rat chow and clean water *ad libitum*.
96 Handling of animals was in accordance with relevant institutional and ethical guidelines
97 as approved for scientific study.

98 **Experimental design**

99 The Wistar rats were randomly assigned into four groups. Details of groupings and
100 treatments are as follows:

101 **Group 1:** Administered with arsenite ($NaAsO_2$) at 1.5 mg/kg body weight.

102 **Group 2:** Administered once with $GdCl_3$ at 2 mg/kg body weight, 24 hours before
103 starting treatment with arsenite ($NaAsO_2$) at 1.5mg/kg of body weight.

104 **Group 3:** Administered once with $GdCl_3$ at 2 mg/kg body weight and subsequently
105 received distilled water.

106 **Group 4:** Administered with distilled water and served as sham control.

107 The arsenite dose was premised on report by Zaki et al (2011). The arsenite dose 1.5
108 mg/kg body weight/day falls within the range of LD_{50} of a 70-Kg body weight human (1 –
109 4 mg/Kg) and lesser than 1/25 of LD_{50} of rats (40 mg/kg). The treatments were daily by
110 oral gavage and lasted for 28 days. Handling of animals was as approved by the
111 Landmark University Research Ethics Committee and consistent with relevant
112 guidelines on the care and use of laboratory animals (National Research Council,
113 2011).

114

115 **Necroscopy**

116 Rats were sacrificed under mild chloroform anesthesia 24 hours after the last
117 treatments. Blood samples were collected into EDTA bottles, centrifuged at 5000 x g for
118 5 minutes in a refrigerated centrifuge (Anke TDL-5000B, Shanghai, China) to obtain the
119 plasma used for the biochemical determinations. The liver from each animal was
120 excised into iced isotonic solution (pH 7.4) and weighed immediately. A fifth of the liver
121 sample was homogenized in iced 0.25 M sucrose solution (1:5, w/v). The liver
122 homogenates were used for the biochemical determinations. The remaining part of the
123 liver was fixed in 10 % buffered neutral formalin (BNF) and used for histopathology
124 examinations.

125

126 **Biochemical assays**

127 The biochemical parameters were determined in rat plasma using a UV/Vis
128 spectrophotometer (Jenway, Staffordshire, United Kingdom) where applicable. The
129 concentration of rat plasma total protein (TP), aspartate aminotransferase (AST – EC:
130 2.6.1.1), alanine aminotransferase (ALT – EC 2.6.1.2), alkaline phosphatase (ALP –
131 EC: 3.1.3.1), γ-Glutamyl transferase (γ-GT – EC 2.3.2.2), bilirubin, albumin, lipid profile
132 including total cholesterol (CHOL), high-density lipoprotein-cholesterol (HDL-C) were
133 determined using Randox assay kits (Crumlin, UK). The low-density lipoprotein
134 cholesterol (LDL-C) was estimated according to the Friedewald formula (Warnick et al.
135 1990). Total protein was determined using the Lowry's method (Lowry et al., 1951).
136 Reduced glutathione level (GSH) was determined by the procedure described by
137 Ellman (1959) and Akanji et al (2009). Malondialdehyde (MDA) was determined using
138 the method described by Niehaus and Samuelson (1968). The concentration of nitric
139 oxide as nitrite/nitrate level was determined using the Greiss method according to
140 method described by Ekanem et al (2009). The myeloperoxidase (MPO – EC 1.11.2.2)
141 activity was determined as previously described (Adeyemi and Sulaiman, 2014),
142 according to the method by Eiserich et al (1998).

143 **Histopathological examination**

144 The rat liver was fixed in 10 % buffered neutral formalin immediately following excision
145 from animals. Fixed tissues were subsequently processed for histopathological
146 examinations as previously described (Adeyemi and Sulaiman, 2012). Capture and
147 scoring for morphological changes were done by a pathologist blind to the treatments,

148 at the University of Ilorin Teaching Hospital, Ilorin, Nigeria. The photomicrographs were
149 captured at x 100 using the software, *Presto! Image Folio package*.

150 **Data analysis**

151 Data were analyzed using the one-way ANOVA (GraphPad Software Inc., San Diego,
152 CA) and presented as the mean ± standard error of mean (SEM). Differences among
153 the group means were determined by the Tukey's test. Mean values at $p<0.05$ were
154 considered to be significant.

155

156

157 **Results**

158 Arsenite exposure had no appreciable effect on rat average weights when compared to
159 the other treatment groups (Fig 1). However, the rat liver weights as well as the liver-to-
160 body weight ratio were significantly reduced by arsenite.

161 Furthermore, oral exposure of rats to arsenite did not alter the concentration of plasma
162 total protein. In contrast, the rat plasma albumin concentration was appreciably
163 decreased relative to the other treatment groups (Fig 2). Arsenic treatment in rats led to
164 a significant elevation of plasma bilirubin concentration relative to the other treatments.

165 In comparison to the other treatment groups, the oral exposure of rats to arsenic for 28
166 days suggest impairment to liver function as evidenced by increased ($p<0.05$) activities
167 of plasma AST, ALT, ALP and γ-GT (Fig 3). Also, arsenic exposure elevated rat plasma
168 and hepatic MDA concentration but depleted the GSH levels when compared to the
169 other treatments (Fig 4). Likewise, arsenic exposure increased the levels of MPO and
170 NO relative to the other treatment groups (Fig 5). The rat plasma lipid profile was not
171 spared, as arsenic exposure elevated the concentration of CHOL, LDL-C but reduced
172 the HDL-C level, thereby increasing the atherogenic index (AI). The negative alterations
173 of plasma lipid profile by arsenic were absent in the other treatment groups (Fig 6).

174 The histopathological assessment of rat liver sections showed inimical cellular lesions
175 including congestion and inflammation caused by arsenic exposure (Fig 7). However,
176 pre-treating rats with $GdCl_3$ before daily exposure to arsenic improved the cellular
177 alterations. In the other groups that received $GdCl_3$ alone and/or distilled water only, the
178 liver cellular architecture was intact.

179

180

181 **Discussion**

182 Arsenic is a toxic metalloid (Aliyu et al., 2012; Das et al., 2012). However, the
183 knowledge of the mechanism of arsenic toxicity remains limited. We reasoned that,
184 identifying the cellular factors that contribute to modulate the response to arsenical
185 toxicity would provide for a better understanding of the mode of toxicity. Kupffer cells
186 have been shown to modulate hepatic injury as well as play key role in the mediation of
187 metal toxicity (Roberts et al., 2007). Therefore, identifying the role Kupffer cells play in
188 arsenic intoxication would aid understanding the mode of toxicity. The present study by
189 using aimed to determine how Kupffer cells affect arsenic intoxication by using $GdCl_3$ to
190 inhibit Kupffer cell functions.

191

192 Arsenic exposure in rats significantly reduced the rat liver weight as well as the liver-to-
193 body weight ratio. This may indicate ensuing adverse effect by arsenite as studies have
194 associated changes to body or organ weights with chemical or drug intoxication
195 (Adeyemi and Orekoya, 2014; Sulaiman et al., 2015). This is consistent with other
196 studies have demonstrated arsenic-induced changes to rat body or organ weights (Aliyu
197 et al., 2012; Chandraayagam et al., 2013).

198 Further evidence for arsenite-induced liver injury is reflected by the negative alteration
199 of the activities of rat plasma AST, ALT, ALP and γ-GT. Increased liver function indices
200 are evidence of liver damage (Adeyemi and Akanji, 2011a; Adeyemi et al., 2012). The
201 arsenic-induced elevation of liver function indices is consistent with the potential to
202 cause liver damage (Gbadegesin and Odunola, 2010; Das et al., Kumar et al., 2014).

203 More so, the arsenite-induced negative alteration of the plasma albumin and bilirubin
204 concentration suggests liver injury or reduced functioning capacity.

205 The arsenic exposure also caused oxidative stress by potentiating lipid peroxidation
206 while depleting the GSH concentrations. GSH plays key role protecting against metal
207 toxicity (Mishra et al., 2008; Swaran et al., 2005) and its depletion may signal oxidative
208 stress (Akanji et al., 2009). In the present study, the use of GSH to detoxify arsenite
209 may have led to the depletion and ensuing oxidative stress observed. Several studies
210 have implicated arsenic for lipid peroxidation, oxidative stress as well as liver damage
211 (Zaki et al., 2011; Chandranayagam et al., 2013). Arsenic's reactivity toward sulfhydryl
212 groups may affect several cellular functions. Studies have demonstrated that arsenite
213 has affinity for thiol groups and could cause damage by promoting generation of
214 reactive species (Rana et al., 2011; Shiobara et al., 2001).

215 The increased level of MPO and NO, consequent of arsenite intoxication may also have
216 aided the lipid peroxidation as well as resultant oxidative cellular damage. Elevated
217 production of NO can react with metals, reduced thiols, molecular oxygen and
218 superoxide leading to adverse reactions (Bryan et al., 2005; Gladwin et al., 2005)
219 leading to cellular damage (Adeyemi and Sulaiman, 2014). Likewise, increased activity
220 of MPO has been linked to oxidative cellular damage (Adeyemi and Akanji, 2011b).
221 MPO plays vital role in neutrophilic inflammation (Vita et al., 2004), and has been shown
222 to potentiate atherosclerosis (Hazen et al., 2004). In like manner, the present study
223 showed that arsenite exposure has capacity to predispose rats to cardiovascular
224 disorder by increasing plasma total CHOL, LDL-C, while lowering the HDL-C levels
225 which raised the atherogenic index. Elevated atherogenic index has been associated

226 with the development of atherosclerosis (Adeyemi and Orekoya, 2014; Olukanni et al.,
227 2013). Our findings agree with studies which have linked arsenic toxicity to
228 cardiovascular disorders (Das et al., 2012; Kumar et al., 2014; Straub et al., 2008).

229 Furthermore, the histopathological assessment of rat liver sections showed cellular
230 lesion caused by arsenite exposure. This supports the biochemical evaluations. Arsenic
231 intoxication has been shown to cause hepatic fibrosis and other degenerating cellular
232 lesions (Abnosi and Jafaru, 2012; Datta et al., 2007; Rana et al., 2011; Zaki et al., 2011)
233 similar to the observation in the present study.

234 However, the pre-treatment of rats with $GdCl_3$ to inhibit the Kupffer cell functions before
235 arsenite exposure suppressed and protected against arsenite intoxication by improving
236 the liver function indices, the oxidative status, lipid profile and ultimately the cellular
237 architecture. The Kupffer cells play central role in the hepatic response to toxicants or
238 carcinogens. The activation of Kupffer cells either directly or indirectly promotes the
239 release of inflammatory mediators as well as reactive oxygen species (ROS) which help
240 modulate hepatic injury. In this study, the inhibition of Kupffer cell functions by $GdCl_3$
241 may have reduced the production of inflammatory mediators including NO and MPO
242 thereby protecting against cellular inflammation and damage. NO and MPO under
243 certain conditions are pro-inflammatory and the duo have been implicated for cellular
244 damage (Adeyemi and Sulaiman, 2014). This suggests that Kupffer cells are a target for
245 arsenic toxicity. The arsenic exposure may have activated the Kupffer cells for
246 protective response that develops to cause cellular damage with further stimulation.
247 This is plausible considering that the inhibition of Kupffer cell functions by $GdCl_3$
248 significantly reduced and improved the indices of arsenite intoxication. Findings in the

249 present study revealed consistence with Roberts et al (2007) which reported that $GdCl_3$
250 inhibition of Kupffer cells could mitigate chemical intoxication as well as resultant
251 cellular damage.

252 **Conclusion**

253 Although, Kupffer cells have dual role in the hepatic response to chemical toxicity, our
254 data supports the complicity of Kupffer cells in arsenite intoxication. To our knowledge,
255 this is the first study showing evidence that inhibiting Kupffer cell functions by using
256 GdCl3 offered protection against arsenite intoxication in rats. Data would contribute to
257 our understanding of the mechanism of arsenite intoxication.

258 **Acknowledgements**

259 Authors acknowledge the laboratory staff at the Department of Biological Sciences for
260 technical support.

261 **Conflict of Interest**

262 Authors declare that there is no conflict of interest.

263

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411

412 **Figure legends**

413 **Figure 1:** Effect of oral exposure to arsenite and/or gadolinium chloride ($GdCl_3$). **A:**
414 Average rat weight **B:** Rat liver weight **C:** Liver-to-body weight ratio. Data are presented
415 as mean value ± standard error of mean (SEM), n = 6. α is significant at p<0.001
416 relative to arsenic only.

417 **Figure 2:** Effect of oral exposure to arsenite and/or gadolinium chloride ($GdCl_3$). **A:** Rat
418 plasma protein concentration. **B:** Rat plasma albumin. **C:** Rat plasma bilirubin. Data are
419 presented as mean value ± standard error of mean (SEM), n = 6. α is significant at
420 p<0.001 relative to arsenic only.

421 **Figure 3:** Effect of oral exposure to arsenite and/or gadolinium chloride ($GdCl_3$). **A:** Rat
422 plasma aspartate transaminase (AST) activity. **B:** Rat plasma alanine transaminase
423 (ALT) activity. **C:** Rat plasma alkaline phosphatase (ALP) activity. **D:** Rat plasma γ-
424 glutamyl transferase (γ-GT) activity. Data are presented as mean value ± standard error
425 of mean (SEM), n = 6. α is significant at p<0.001 relative to arsenic only.

426 **Figure 4:** Effect of oral exposure to arsenite and/or gadolinium chloride ($GdCl_3$). **A:** Rat
427 plasma malondialdehyde (MDA) concentration **B:** Rat hepatic malondialdehyde (MDA)
428 concentration. **C:** Rat plasma reduced glutathione (GSH) concentration. **D:** Rat hepatic
429 reduced glutathione (GSH) concentration. Data are presented as mean value ±
430 standard error of mean (SEM), n = 6. α is significant at p<0.001 relative to arsenic only.

431 **Figure 5:** Effect of oral exposure to arsenite and/or gadolinium chloride ($GdCl_3$). **A:** Rat
432 plasma nitric oxide (NO) concentration measured as nitrate/nitrite level. **B:** Rat plasma
433 myeloperoxidase (MPO) activity. Data are presented as mean value ± standard error of
434 mean (SEM), n = 6. α is significant at p<0.001 relative to arsenic only.

435 **Figure 6:** Effect of oral exposure to arsenite and/or gadolinium chloride ($GdCl_3$). **A:** Rat
436 plasma total cholesterol concentration. **B:** Rat plasma high density lipoprotein-
437 cholesterol (HDL-C) concentration. **C:** Rat plasma low density lipoprotein-cholesterol
438 (LDL-C) concentration. **D:** Rat atherogenic index (AI – LDL/HDL ratio). Data are
439 presented as mean value ± standard error of mean (SEM), n = 6. α is significant at
440 p<0.001 relative to arsenic only.

441 **Figure 7:** Photomicrographs of rat liver following exposure to arsenic and/or gadolinium
442 chloride ($GdCl_3$). **A:** Arsenic treatment only – showing distortion of the cellular
443 architecture with a severe congestion of the central vein. **B:** Arsenic and gadolinium
444 chloride treatment - showing a slight distortion of the cellular architecture with a mild
445 congestion of the central vein. **C:** Gadolinium chloride treatment only - showing a well
446 preserved cellular morphology with a free central vein. **D:** Normal saline **–** showing a
447 well preserved cellular architecture with a free central vein. H & E Stain. (x400).

448

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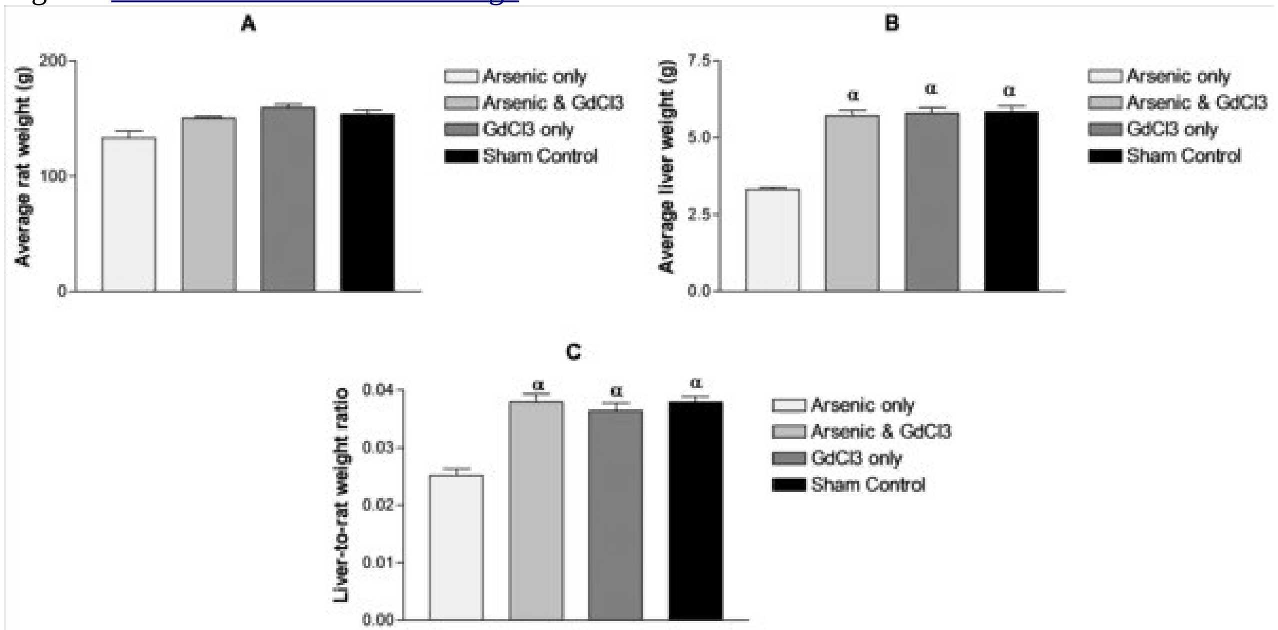

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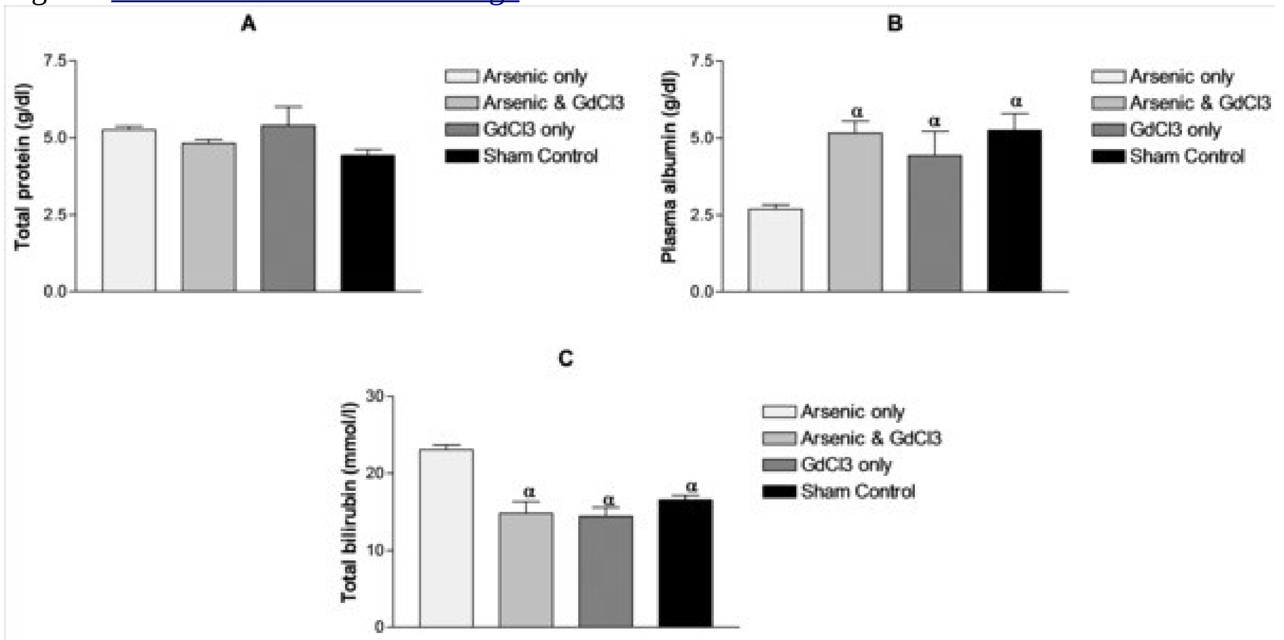

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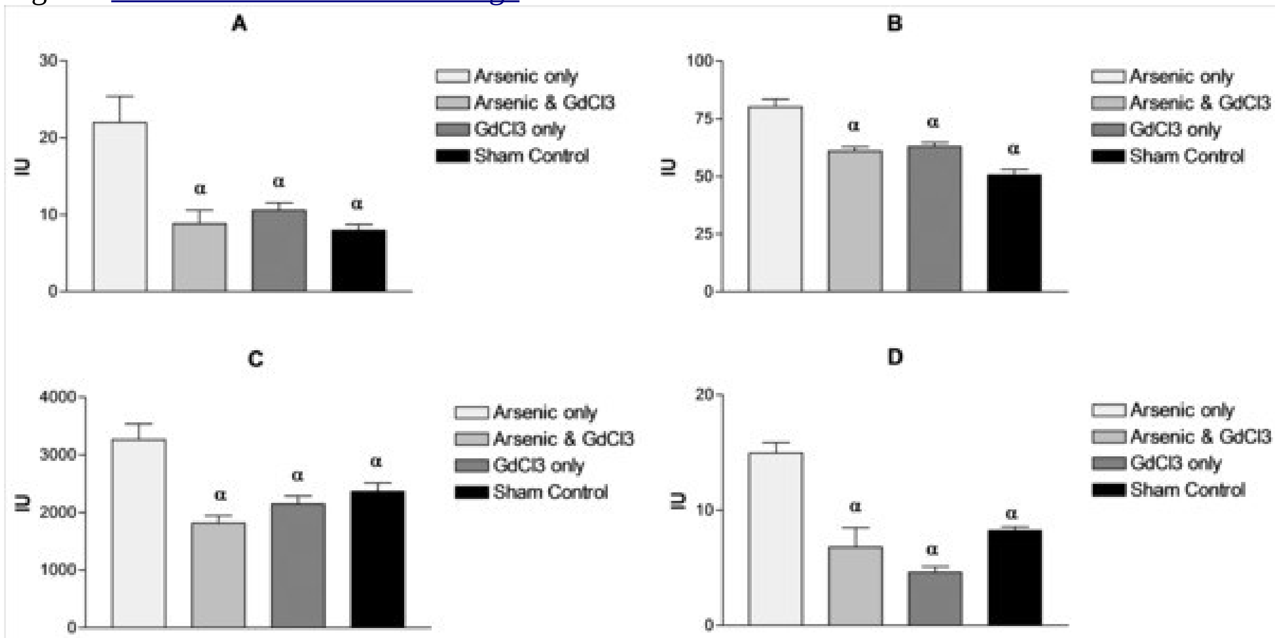

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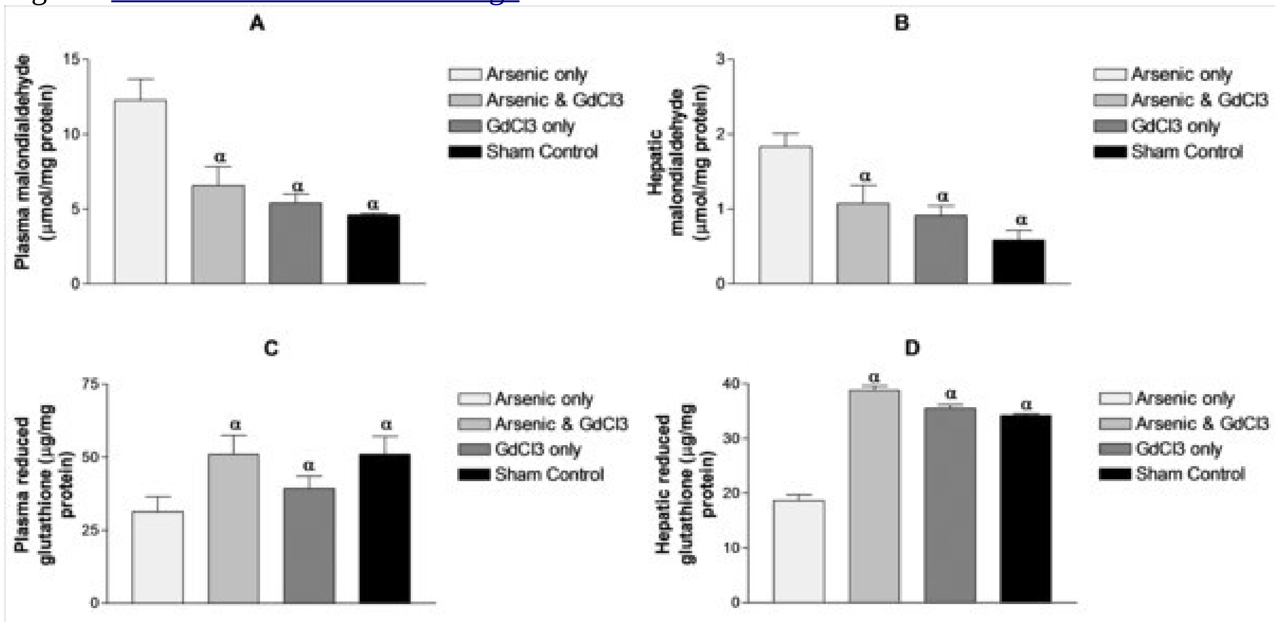

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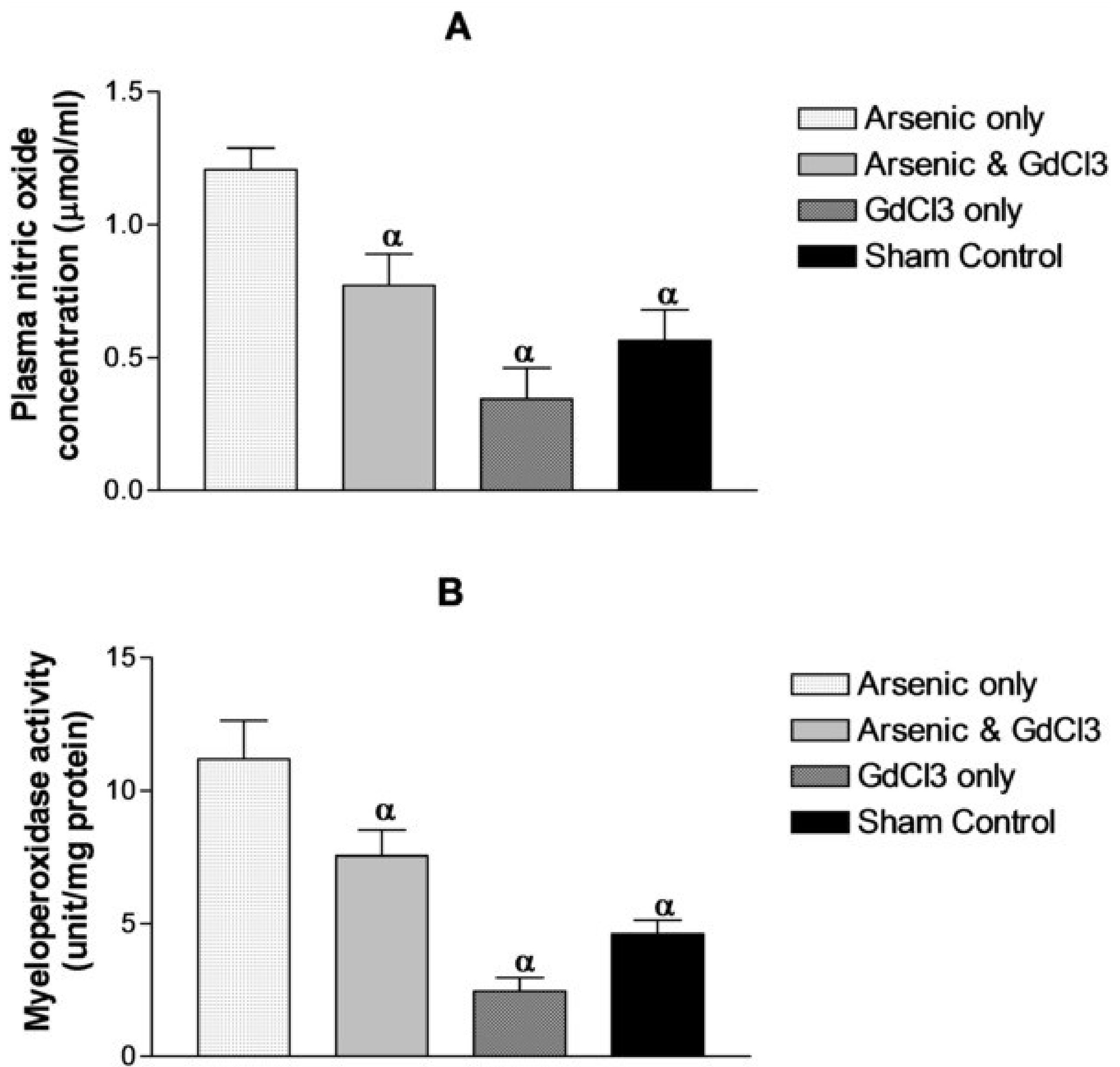

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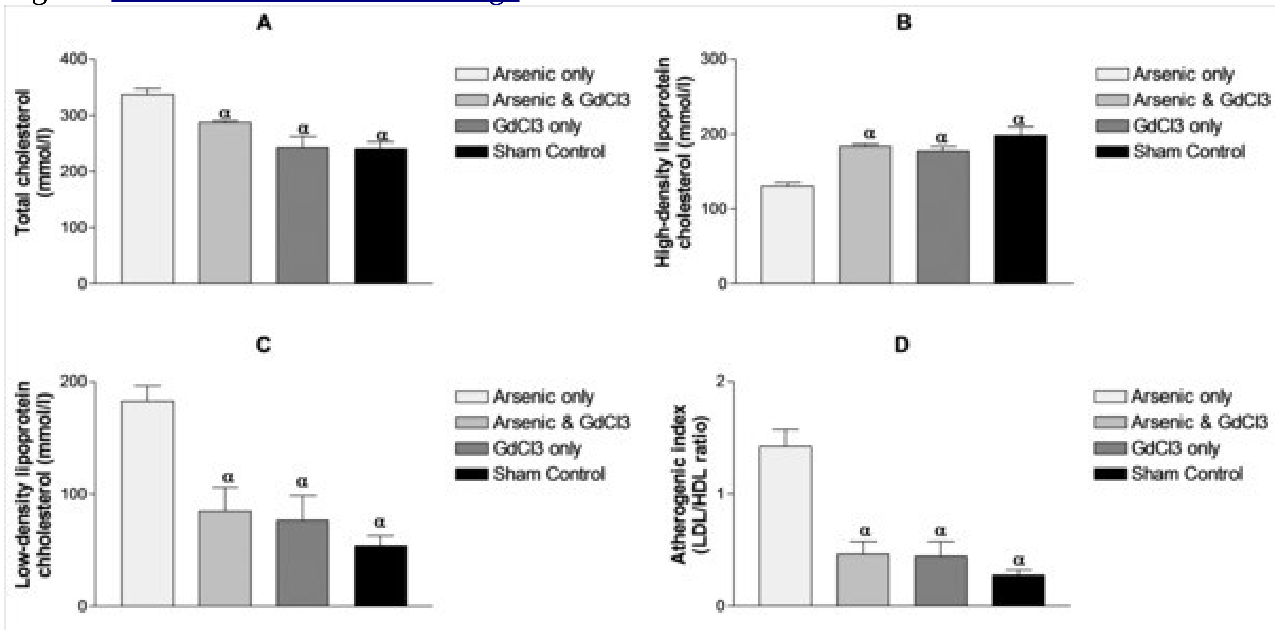

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