

1 **Short communication**

2 **A therapeutic oxygen carrier isolated from *Arenicola marina* decreases amanitin-**
3 **induced hepatotoxicity**

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25 **ABSTRACT**

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27 The amanitins (namely α - and β -amanitin) contained in certain mushrooms are bicyclic octapeptides
28 that, when ingested, are responsible for potentially lethal hepatotoxicity. M101 is an extracellular
29 hemoglobin extracted from the marine worm *Arenicola marina*. It has intrinsic Cu/Zn-SOD-like
30 activity and is currently used as an oxygen carrier in organ preservation solutions. Our present
31 results suggest that M101 might be effective in reducing amanitin-induced hepatotoxicity and
32 may have potential for therapeutic development.

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36 **KEYWORDS:** amanitin, oxygen carrier, M101, hepatocyte, hepatotoxicity

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46 1. INTRODUCTION

47 Amanitins are high-molecular-weight bicyclic octapeptides present in certain mushrooms. When
48 ingested, these compounds are responsible for potentially lethal hepatotoxicity. The best-characterized
49 amanitins are α -amanitin and β -amanitin because they are present at high levels in the mushrooms
50 involved in poisonings (Kaya et al., 2013). After amanitin accumulation in the liver *via* uptake through
51 the organic anion transporter OATP1B3 in the hepatocyte's sinusoidal membrane (Letschert et al.,
52 2006), the compounds inhibit the RNA polymerase II in the nucleus and thus jeopardize mRNA
53 translation and protein synthesis (Wieland, 2009). The amanitin-induced stress signal has been shown
54 to induce p53 protein, which then forms complexes with protective proteins (Bcl-X_L and Bcl-2) and
55 subsequent triggers apoptosis via release of mitochondrial cytochrome c into the cytosol (Arima et al.,
56 2005; Leu and George, 2007; Ljungman et al., 1999). Accordingly, Wang et al. (2018) showed that α -
57 amanitin induces significant changes in the mitochondrial proteome and might destroy the
58 mitochondrial membrane potential (Wang et al., 2018). It has also been suggested that oxidative stress
59 is important in the development of severe hepatotoxicity, since α -amanitin accumulation leads to an
60 increase in the activity of superoxide dismutase (SOD) and glutathione peroxidase, the production of
61 malondialdehyde, and lipid peroxidation, and inhibits catalase activity (DüNdar et al., 2017; Zheleva
62 et al., 2007). Furthermore, it has been reported that α -amanitin forms phenoxyl free radicals that might
63 be involved in the production of reactive oxygen species (ROS) (Zheleva, 2013). Interestingly, most
64 effective antidotes (including N-acetylcysteine, silymarin and silibinin) have antioxidant properties
65 (Magdalan et al., 2011; Tavassoli et al., 2019; Ye and Liu, 2018). Thus, avoiding oxidative stress
66 appears to be the most promising treatment option.

67 M101 is an extracellular hemoglobin extracted from the marine worm *Arenicola marina*. It is
68 currently used in medicine as an oxygen carrier in organ preservation solutions. This class III medical
69 device (commercialized as HEMO₂life[®] by Hemarina, Morlaix, France) was found to improve the
70 preservation of kidney grafts (Mallet et al., 2014; Thuillier et al., 2019), hearts (Teh et al., 2017) lungs
71 (Ali et al., 2020; Glorion et al., 2017) and liver transplants (Alix et al., 2020) *in vivo*. Furthermore, *in*
72 *vitro* studies have demonstrated that M101's intrinsic Cu/Zn-SOD-like activity can protect against the
73 damage potentially caused by ROS release (Lemaire et al., 2019; Rousselot et al., 2006; Thuillier et
74 al., 2019). Moreover, M101's good safety profile has been demonstrated in preclinical studies -

75 prompting its therapeutic development. In this context, the objective of the present study was to
76 evaluate M101's effect on the putative mechanisms of amanitin-induced hepatotoxicity and thus its
77 potential for therapeutic development.

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79 **2. MATERIAL AND METHODS**

80 **2.1 Reagents:** Phosphate-buffered saline (PBS), Williams's E medium, Hanks' balanced salt solution
81 (HBSS), penicillin-streptomycin, and L-glutamine were purchased from Life Technologies (Eugene,
82 OR, USA). Fetal calf serum (FCS) was obtained from Hyclone (Logan, UT, USA). Insulin,
83 hydrocortisone, α - and β -amanitin, **SOD inhibitors (diethylthiocarbamic acid [DETC] and 1,4,5-**
84 **dichloro-2-m-tolylpyridazin-3(2H)-one [LCS]) and SOD assay kit** were from Sigma Aldrich (St.
85 Louis, MO, USA). The luminescent ATP detection assay kit was purchased from Abcam (Cambridge,
86 MA, USA). MitoSOX red mitochondrial superoxide indicator was from ThermoFisher Scientific
87 (Waltham, MA, USA). M101 (HEMO₂life[®]) was provided by Hemarina as a frozen stock solution (50
88 g/L) and used in these experiments at a final concentration in culture medium of 1 g/L (the
89 concentration used in organ preservation solutions). **Carboxy-M101 (M101-CO) was obtained by**
90 **bubbling carbon monoxide gas 99.997% (Rapid'Gaz, Verdun, France) for 10 minutes in the M101**
91 **solution.**

92 **2.2 Cell cultures:** Progenitor HepaRG cells were cultured as described elsewhere (Aninat et al., 2006).
93 Briefly, the cells were seeded at a density of $2.6 \times 10^4/\text{cm}^2$ in 96-well plates in William's E medium
94 supplemented with 10% FBS, 50 U/mL penicillin, 50 $\mu\text{g}/\text{mL}$ streptomycin, 5 $\mu\text{g}/\text{mL}$ insulin, 2 mM
95 glutamine, and 50 μM sodium hydrocortisone hemisuccinate. After two weeks, cells were cultured for
96 a further two weeks in the same medium supplemented with 2% DMSO in order to promote
97 hepatocyte differentiation into both cholangiocyte- and hepatocyte-like cells (Cerec et al., 2007).

98 **2.3 Measurement of amanitin-induced mitochondrial stress:** MitoSOX Red Mitochondrial
99 Superoxide Indicator (ThermoFisher Scientific, MA, USA) was used to measure mitochondrial ROS
100 production. Briefly, cells were incubated in the dark with 100 μL of MitoSOX (5 μM) probes for 30
101 minutes at 37°C and with 5% CO₂. After the required incubation time, the medium was removed and
102 the cells were washed once with 100 μL of HBSS at room temperature. The wells were then read
103 quickly using a fluorescence microplate reader (POLARstar Omega®, BMG labtech, Ortenberg,

104 Germany), and the data were analyzed using MARS software (BMG labtech). The results were
105 normalized for the protein concentration assay (determined with a PierceTM BCA protein assay Kit,
106 (ThermoFisher Scientific).

107 **2.4 Cell viability:** Cytotoxicity was assessed using a Luminescent ATP Detection Assay Kit (Abcam),
108 according to the manufacturer's instructions. Plates were read using a microplate reader (POLARstar
109 Omega[®], BMG labtech).

110 **2.5 SOD activity measurement:** SOD activity was assessed using SOD assay kit (Sigma Aldrich, St.
111 Louis, MO, USA) according to the manufacturer's instructions. HepaRG cell lysis was performed with
112 RIPA buffer (Sigma Aldrich) and SOD activity was evaluated in 40 μ l of cell lysate.

113 **2.6 Statistical analysis:** Data were expressed as the mean \pm standard error of the mean (SEM).
114 Intergroup differences as a function of the treatment were probed in a one-way analysis of variance
115 (ANOVA), with a Bonferroni *post hoc* test for group comparisons. All analyses were performed using
116 Prism software (version 5.0, GraphPad Software, La Jolla, CA, USA). All tests were two-sided, and
117 the threshold for statistical significance was set to $p < 0.05$. Differences between α - and β -amanitin
118 were probed in a two-way ANOVA, with a bonferroni *post hoc* test.

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120 **3. RESULTS**

121 Since amanitin exposure is known to be hepatotoxic, we looked at whether these toxins were able to
122 dose-dependently decrease viability in a cell-based model of the liver (i.e. differentiated HepaRG
123 cells). We found that amanitin significantly decreased the viability of differentiated HepaRG cells in a
124 dose-dependent manner, starting at 1 μ M - showing that the model was sensitive (Figure 1A). α -
125 amanitin was more potent than β -amanitin in terms of loss of viability at 1 μ M, but not at other
126 concentrations. Furthermore, we observed that α - or β -amanitin increased mitochondrial ROS
127 generation in a dose-dependent manner, starting at 2 μ M. Mitochondrial ROS production was also
128 higher with α -amanitin compared to β -amanitin at 2 μ M ($p < 0.05$), but not at other concentrations.
129 Taken together, these results indicate that the amanitins' toxic effect on differentiated HepaRG cells is
130 dependent - at least in part - on mitochondrial ROS production.

131 In order to investigate M101's effect on amanitin-induced toxicity, all subsequent experiments were
132 performed with the concentration of α - and β -amanitin (2 μ M) that reduced the viability of

133 differentiated HepaRG cells by 50% and induced significant mitochondrial oxidative stress. M101 (1
134 g/L) was added at the same time as amanitins (H0), 1 h after amanitins (H1), 3 h after amanitins (H3)
135 or 6 h after amanitins (H6). **Under all conditions, M101 remained incubated along with amanitins.** We
136 found that M101 alone (1 g/L) significantly increased cell viability. We observed that after 24 h of
137 exposure to amanitin, cell viability was significantly higher in the presence of M101 (1 g/L) when
138 added up to 6 h after β -amanitin (H6) and up to 3 h after α -amanitin (H3) (Figure 2A). This effect was
139 associated with a significantly lower level of mitochondrial ROS generation in the presence of M101
140 (1 g/L) when added at the same time as α -amanitin (H0) and up to 6 h after β -amanitin (H6) (Figure
141 2B). M101 resulted in an increase in cell viability as well as a greater decrease in ROS production
142 with β -amanitin than with α -amanitin. Taken together, these results show that M101 protects a cell-
143 based hepatic model against amanitin's toxicity. Furthermore, M101 had a protective effect of the cells
144 even when added several hours after amanitin.

145 **Since M101 is capable of delivering oxygen to the surrounding environment, we aimed to evaluate**
146 **whether M101 without its oxygen carrier proprieties (M101-CO) impacted intracellular SOD activity.**
147 **HepaRG cells were incubated with M101 (1 g/L) or M101-CO (1 g/L) with or without SOD1**
148 **inhibitors (DETC 100 μ M or LCS 10 μ M) (Figure 2C). We found that M101 was able to increase**
149 **intracellular SOD activity ($p < 0.01$), even in the presence of DETC ($p < 0.01$). Similar trend was**
150 **observed using LCS without reaching statistical significance. This effect was no longer present using**
151 **M101-CO (Figure 2C). Interestingly, none of these conditions resulted in cell death (Figure 2D).**

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153 **4. DISCUSSION**

154 In this study, we found that a therapeutic oxygen carrier isolated from *Arenicola marina* (M101) had
155 protective effect on amanitin-induced toxicity on *in vitro* hepatic cell model. We first evaluated the
156 amanitins' toxicity on differentiated HepaRG cells *in vitro*; we observed a dose-dependent decrease in
157 cell viability and a concomitant elevation in mitochondrial ROS generation, starting at an amanitin
158 concentration of 2 μ M. Therefore, this 2 μ M dose was particularly relevant for testing the efficacy of
159 an antidote on these two parameters. These results are in line with the literature data on the
160 involvement of oxidative stress in amanitin toxicity (DüNdar et al., 2017; Zheleva, 2013; Zheleva et
161 al., 2007). Our study showed that differentiated HepaRG cells constitute a sensitive, reproducible

162 model for studying the toxicity of amanitin. We found that M101 alone significantly increased cell
163 viability, probably because M101 has oxygen-carrier properties, resulting in increased mitochondrial
164 activity. Interestingly, M101 was able to restore HepaRG cell viability and decrease mitochondrial
165 ROS generation after α - and β -amanitin exposure. However, it is unknown whether a change in cell
166 viability was due to an increase in ROS production or the changes in ROS was due to reduced
167 viability. Moreover, we showed that the cells' ROS production was greater after α -amanitin exposure
168 than after β -amanitin exposure. This might explain why (i) M101 had a greater effect on β -amanitin-
169 induced effects and (ii) cell viability was even restored when M101 was added several hours after the
170 toxin. Given that α - and β -amanitin are neutral and acid compounds, one can reasonably hypothesize
171 that the differing responses to M101 are related to the toxins' physical-chemical properties (Garcia et
172 al., 2015). Considering M101's macrostructure (3600 kDa, 15 x 25 nm), this hemoglobin is unlikely to
173 enter hepatocytes. However, we hypothesize that M101's ~~oxygen-carrier antioxidant-Cu/Zn-SOD-like~~
174 properties might be ~~partially~~ responsible for the beneficial effect observed *in vitro*. ~~Using M101-CO,~~
175 ~~which allowed us to observe the effects of M101 without its oxygen-carrier properties (G. Tsai et al.,~~
176 ~~2012), we showed that M101 lost its intracellular SOD activating effect. Thus, although the precise~~
177 ~~mechanisms are not elucidated, these results suggest that oxygen supply by M101 is an important~~
178 ~~feature in its hepatoprotective effect. This does not exclude that other effects may be part the~~
179 ~~mechanism. For example, another M101 effect on HepaRG cells might be the down-regulation of pro-~~
180 ~~inflammatory cytokines and chemokine ligands, or the upregulation of pro-healing mediators and~~
181 ~~immune modulators, as already reported (Batoool et al., 2020). Lastly, further studies would be needed~~
182 ~~to determine whether M101's antioxidant Cu/Zn SOD-like properties are involved (Rousselot et al.,~~
183 ~~2006).~~

184 Our present results can be considered with regard to the currently available treatments for *Amanita*
185 *phalloides* poisoning. The death rate associated with this poisoning is still high (between 10% and
186 20%), despite the use of antidotes such as ~~penicillins~~, thioctic acid, N-acetylcysteine, vitamin C,
187 silymarin, and silibinin (Enjalbert et al., 2002; Escudié et al., 2007; Ganzert et al., 2005; Le Daré et
188 al., 2021). The fact that none of these compounds are highly effective means that *Amanita phalloides*
189 poisoning is still a challenging medical emergency. We consider that there are two therapeutic

190 perspectives for the use of M101. Firstly, intravenous infusion can be considered, since
191 biodistribution studies in mice have shown that M101 reaches the liver easily (Le Gall et al.,
192 2014). It has also been reported that silymarin enhances SOD activity, which enables a fall in
193 oxidative stress through the harvesting and recycling of ROS, and thus an overall decrease in
194 tissue inflammation and cell death. We suggest that M101's SOD-like activity (alone or in
195 combination with currently available antidotes) might be of therapeutic value for promoting
196 hepatocyte survival (Wellington and Jarvis, 2001). Secondly, M101 could be added to an
197 organ preservation solution if liver transplantation is required after *Amanita phalloides*
198 poisoning. Although this requirement is infrequent, there are case reports of re-intoxication of
199 the newly transplanted graft – jeopardizing the clinical outcome (Kucuk et al., 2005).
200 Furthermore, this hemoglobin was developed first in the organ preservation indication, and its
201 good safety profile has been reported in many indications - including liver transplant (Alix et
202 al., 2020; Glorion et al., 2017; Mallet et al., 2014; Teh et al., 2017; Thuillier et al., 2019).
203 Lastly, Ye et al. (2018) reported that liver transplantation was considered to be the only
204 approach for guaranteeing survival in *Amanita phalloides* poisoning with fulminant hepatic
205 failure (Ye and Liu, 2018). Our present data suggest that a liver transplant incubated with
206 M101 might offer a better outcome in this context.

207 In conclusion, the present study is the first to have demonstrated the value of M101 for
208 reducing amanitin-induced toxicity in an *in vitro* hepatic cell model. Further studies are
209 needed to explore the exact mechanisms involved in M101's hepatoprotective effects. *In vivo*
210 studies would be of particular interest.

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213 **Contributors**

214 All authors contributed significantly, and all authors agree with the content of the manuscript:

215 Conception/Design: Brendan Le Daré, Thomas Gicquel, Vincent Lagente

216 Collection and/or assembly of data: Brendan Le Daré, Thomas Gicquel, Vincent Lagente, Nessrine
217 Bellamri, Pierre-Jean Ferron, Catherine Ribault
218 Data analysis and interpretation: All authors
219 Manuscript writing: All authors
220 Final approval of manuscript: All authors.

221

222 **Conflict of Interest Statement:**

223 Franck Zal is the founder of and holds stock in Hemarina, the company that produces M101 and
224 commercializes it as HEMO₂life[®]. Eric Delpy is an employee of Hemarina. The other authors declare
225 that they have no conflicts of interest.

226

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252 **inflammation and tissue destruction. *Sci. Rep.* 10, 14745. [https://doi.org/10.1038/s41598-020-](https://doi.org/10.1038/s41598-020-71593-8)**
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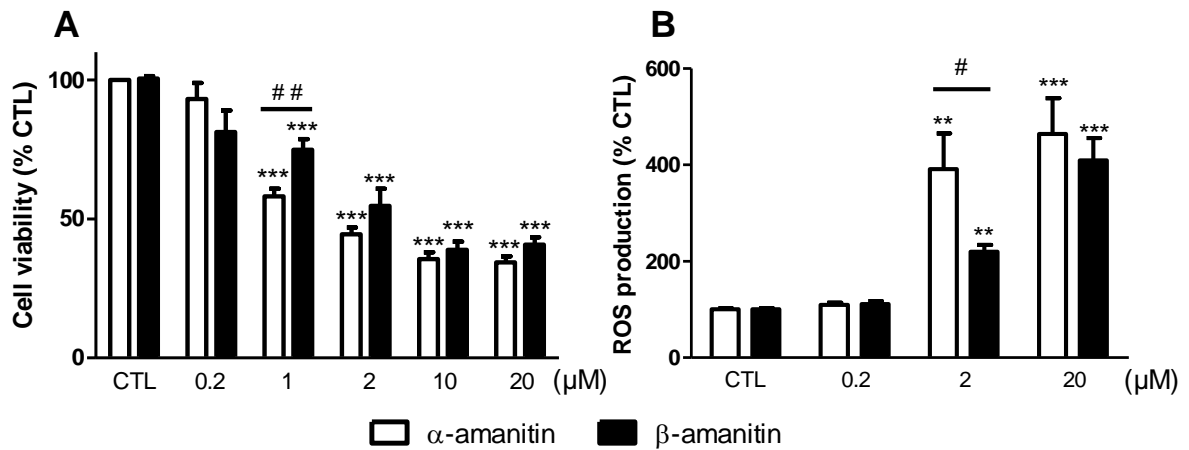
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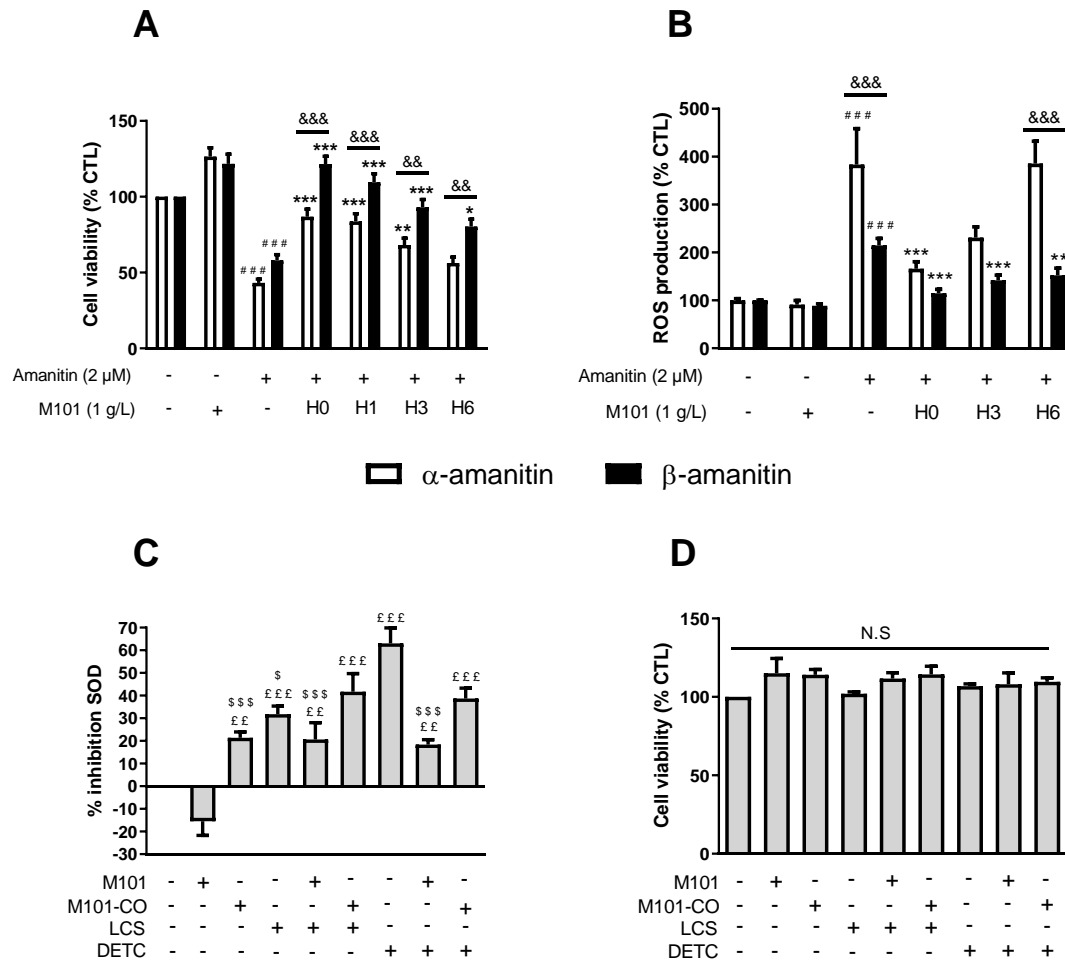
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354 **Figure 1: Effect of α - and β -amanitin on the viability of differentiated hepatocytes.** Differentiated
 355 HepaRG cells were cultured with α - or β -amanitin (0.2 to 20 μ M) or medium for 24 h. Cell viability
 356 was measured using an extracellular ATP assay and expressed relative to the value determined after
 357 treatment with medium alone (arbitrarily set to 100%). Levels of mitochondrial ROS 24 h after
 358 amanitin treatment were detected using a MitoSOX (5 μ M) probe and normalized against the protein
 359 content. The data are quoted as the mean \pm SEM from at least three independent experiments
 360 performed in triplicate. ** $p < 0.01$ *** $p < 0.001$: the control condition compared with α - or β -
 361 amanitin at different concentrations. # $p < 0.05$ ## $p < 0.01$: α -amanitin compared with β -amanitin.

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374 **Figure 2: Effect of M101 on amanitin-induced hepatotoxicity.** (A and B) Differentiated HepaRG

375 cells were cultured with α - or β -amanitin (2 μ M) or medium for 24 h at 37°C and with 5% CO₂ in the

376 absence or presence of M101 (1 g/L) added up to 6 h after amanitin treatment. (A and D) The viability

377 of differentiated HepaRG cells was measured using an extracellular ATP assay and expressed relative

378 to the value determined after treatment with medium alone (arbitrarily set to 100%). (B) Levels of

379 mitochondrial ROS 24 h after amanitin treatment were detected using a MitoSOX (5 μ M) probe and

380 normalized against the protein content. (C) SOD activity in the presence of M101 (1 g/L), M101-CO

381 (1 g/L), DETC (100 μ M) and/or LCS (10 μ M) was determined using SOD assay kit and expressed

382 relative to the value determined after treatment with medium alone (arbitrary set to 0%). The data are

383 quoted as the mean \pm SEM from three independent experiments performed in triplicate. ## $p < 0.01$ ###

384 $p < 0.001$: Control condition compared with α - or β -amanitin alone. * $p < 0.05$ ** $p < 0.01$ *** $p <$

385 0.001: α - or β -amanitin alone compared with α - or β -amanitin + M101 added at a different time point

386 (H0, H1, H3, H6). && p < 0.01 &&& p < 0.001 : α -amanitin compared with β -amanitin. \$ p < 0.05
387 \$\$\$ p < 0.001: in comparison with DECT alone. ££ p < 0.01 £££ p < 0.001: in comparison with M101
388 alone.
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