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1   **Porcine small and large intestinal microbiota rapidly hydrolyze the masked mycotoxin**

2   **deoxynivalenol-3-glucoside and release deoxynivalenol in spiked batch cultures *in vitro*.**

3   Running title: Porcine intestinal microbiota hydrolyze DON3Glc

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13 trichothecene

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17

18   Abstract

19   Mycotoxin contamination of cereal grains causes well-recognized toxicities in animals and

20   humans, but the fate of plant-bound masked mycotoxins in the gut is less well understood.

21   Masked mycotoxins have been found to be stable under conditions prevailing in the small

22   intestine, but are rapidly hydrolyzed by fecal microbiota. This study aims to assess the

23   hydrolysis of the masked mycotoxin deoxynivalenol-3-glucoside (DON3Glc) by microbiota of

24   different regions of the porcine intestinal tract.

25   Intestinal digesta samples were collected from the jejunum, ileum, caecum, colon and feces of

26   5 pigs and immediately frozen under anaerobic conditions. Sample slurries were prepared in

27   M2 culture medium, spiked with DON3Glc or free DON (2 nmoles/mL) and incubated

28   anaerobically for up to 72 hours. Mycotoxin concentrations were determined using LC-MS/MS

29   and microbiota composition was determined using qPCR methodology.

30   Jejunal microbiota hydrolyzed DON3Glc very slowly, while samples from the ileum, caecum,

31   colon and feces rapidly and efficiently hydrolyzed DON3Glc. No further metabolism of DON

32   was observed in any sample. Microbial load and microbiota composition was significantly

33   different in the ileum, but similar in caecum, colon and feces.

34

35   Importance

36   Results from this study clearly demonstrate that the masked mycotoxin DON3Glc is

37   hydrolyzed efficiently in the distal small intestine and large intestine of pigs. Once DON is

38   released, toxicity and absorption in the distal intestinal tract are likely to occur *in vivo*. This

39   study further supports the need to include masked metabolites into mycotoxin risk assessments

40   and regulatory actions for feed and food.

41

42   Introduction

43   Mycotoxin contamination of agricultural commodities is an intractable problem globally. In

44   temperate climates *Fusarium* fungi comprise the most important mycotoxin producers and are

45   particularly prevalent in small grain cereals such as wheat and barley as well as maize. The

46   major groups of *Fusarium* mycotoxins include trichothecenes, zearalenone and fumonisins [1].

47   In addition to the well described trichothecenes deoxynivalenol (DON), nivalenol, T2 toxin

48   and HT2 toxin, cereals have been found to be co-contaminated with plant-derived mycotoxin

49   metabolites, so-called masked mycotoxins. In response to fungal infection and mycotoxin

50   production, the plant's own phase II metabolic enzymes conjugate mycotoxins with small

51   molecules such as glucose, glutathione or sulphate and sequester these masked mycotoxins into

52   the plant cell vacuole (for review see [2-4]). Mycotoxins and masked mycotoxins are stable

53   compounds withstanding processing into various cereal products and are carried over into

54   finished food and feed. Once ingested, mycotoxins have been shown to be rapidly absorbed in

55   the small intestine of humans and various animal species and exert their toxicities either locally

56   on the gut epithelium (e.g. trichothecenes) or systemically (e. g. zearalenone) [1,4-6]. Masked

57   mycotoxins such as DON-3-β,D-glucoside (DON3Glc) on the other hand, are far less toxic

58   compared to their free parent mycotoxins and are not absorbed intact [7-9]. Hence masked

59   mycotoxins are transported into the distal parts of the intestine intact where the intestinal

60   microbiota (as studied using fecal samples) rapidly hydrolyze masked mycotoxins and release

61   free mycotoxins [7,10-12]. Microbial metabolism experiments have also demonstrated further

62   metabolism of DON to de-epoxy DON (DOM-1) by microbiota samples derived from

63   chickens, pigs, and some humans [10,13,14]. This purely microbial metabolite, DOM-1, is not

64   toxic [15] and can be found in urine of some humans [10,16,17] and pigs [18] hence confirming

65   its production and colonic absorption i*n vivo*.

66 In pigs oral bioavailability and absorption of DON3Glc is significantly lower and slower,

67 compared to DON [18,19]. The delay in DON3Glc absorption and the fact that only free DON

68 and no DON3Glc are found in plasma and urine, confirm that the hydrolysis and absorption

69 occur in the more distal parts of the intestinal tract compared to free DON. Microbial de-

70 epoxidation of DON or DON3Glc by pig microbiota has been found in some studies [14,18]

71 but not in others [19].

72 All studies published to date have used fecal samples from pigs or humans to determine

73 microbial hydrolysis and metabolism of mycotoxins. However, microbial metabolism of

74 mycotoxins would need to occur in more proximal parts of the intestinal tract to release

75 mycotoxin metabolites and allow intestinal absorption and/or potential colonic toxicity to

76 occur. Therefore, the aim of this study was to investigate the capacity of intestinal microbiota

77 derived from different regions of the small and large intestine of pigs to degrade masked

78 mycotoxins. For this study, DON3Glc was used as model mycotoxin as it is commercially

79 available.

80

81 Materials and Methods

82 The following mycotoxin standards were used in this study: DON as powder (Molekula,

83 Gillingham, UK); DON, and DON3Glc in acetonitrile (Romer Labs, Runcorn, UK) and DOM-

84 1 in acetonitrile (Sigma-Aldrich Ltd, Poole, UK).

85

86 *Animals and ethical approval*

87 Five crossbred castrated male pigs, weaned at four weeks were bred in the animal facility of

88 the INRA ToxAlim Laboratory (Toulouse, France). The experiment was conducted under the

89   authorization of the French ministry of Higher Education and Research after approval by the

90   Ethics committee of Pharmacology-Toxicology of Toulouse-Midi-Pyrénées (Toxcométhique,

91   N°: TOXCOM/0163 PP), in accordance with the European Directive (2010/63/EU) on the

92   protection of animals used for scientific purposes. Feed and water were provided ad libitum

93   throughout the experimental period. Pigs were fed for 4 days with starter diet and then with a

94   commercial diet "STIMIO" for growing pigs (Evialis, Longue Jumelles, France), the feed

95   composition is summarized in Table 1. As the presence of antibiotics or probiotics in feed can

96   alter the composition of the luminal and mucosa-associated microbiota [20] non-supplemented

97   feed was used. Pigs were maintained until 57 days of age as the pig intestinal flora is stable

98   between at least 48 and 70 days of age [21]. Then, they were subjected to electronarcosis and

99   euthanized by exsanguination [22]. The intestinal tract was removed from each carcass and

100   sections of the jejunum, ileum, caecum and colon were dissected. Five millilitres of intestinal

101   digesta content from each gut section was collected separately into sterile Wheaton bottles.

102   Feces (5 mL) was sampled directly from the pen. Ten mL of a sterile mixture of 70% phosphate

103   buffered saline (pH 7.4) /30% glycerol bubbled with CO_2 were added into each vial. Vials were

104   sealed and the headspace flushed with CO_2 before being stored at -20°C.

105

106   *Microbial batch culture experiments*

107   After defrosting and vortexing, 15 mL of slurry were centrifuged at $2000 \times g$ for 5 minutes.

108   Supernatant was discarded and the remaining pellet was purged with CO_2. At this stage, two 1

109   mL aliquots were removed from each sample and stored in sample Matrix tubes at -70°C for

110   subsequent DNA extraction. The remaining slurry was diluted 1/10 with anaerobic M2 medium

111   as described before [10], placed in a shaking water bath (37°C, 100 rpm) in a sealed Wheaton

112   bottle for 1 hour and 1 mL aliquots were moved to sterile screw-capped Hungate tubes. Slurry

113   aliquots were spiked with individual mycotoxins (2 nmol/mL of DON, DON3Glc or DOM-1)

114   and incubated anaerobically at 37°C for intervals between 0-72 hours. This wide range of

115   incubation times was chosen to reflect both the short transit time in the small intestine (early

116   time points) and the long transit time in the large intestine (late time points). Following

117   incubation, 3 mL acetonitrile were added to each sample and samples were centrifuged for 5

118   minutes at $2000 \times g$. Supernatants were evaporated under N_2 at 50°C, reconstituted with 1 mL

119   of water and passed through C18 solid phase extraction columns (Agilent, Wokingham, UK).

120   Samples were eluted with 3 mL methanol, evaporated under N_2 at 50°C, reconstituted into 1

121   mL of 50% aqueous methanol, and analysed for DON, DON3Glc and DOM-1 using LC-

122   MS/MS. Blank digesta incubations (omitting spiking with mycotoxins) were included in each

123   experiment to ensure that all digesta samples were free of mycotoxin residues. Furthermore,

124   DON3Glc or DON (2 nmoles/mL) were spiked individually into bacteria-free M2 culture

125   media (in duplicates) and incubated for 72 hours to ensure stability of DON3Glc and DON

126   under incubation conditions in the absence of bacteria. Both compounds were stable with

127   recoveries of 100.7± 4.7% and 102.8± 1.9%, respectively after 72 hours. To further assess

128   DON stability during incubation, digesta samples were spiked with DON (2 nmoles/mL) and

129   incubated for 0 to 72 hours. This experiment showed no mass loss of DON (recovery up to

130   119% of dose added), suggesting no binding of DON or further metabolism by microbes or

131   any other digesta constituents. Each experiment also included digesta controls (in duplicate)

132   spiked with DON3Glc, DON or DOM-1 individually, which were not incubated and

133   immediately processed further (i.e. time 0) to account for potential matrix effects in mycotoxin

134   detection. Mycotoxins detected in time 0 samples were set as 100% and all other results were

135   calculated as % of time 0.

136

137   *QPCR analysis of microbial composition*

138   Microbiota composition was analyzed using DNA extracted from untreated digesta samples

139   (without mycotoxin spiking) derived from ileum, caecum, colon and feces of experimental

140   pigs. Jejunal samples did not yield sufficient DNA to perform qPCR analysis. DNA was

141   extracted from 1 mL of digesta slurry using the FastDNA™ Kit for Soil, (MP Biomedicals,

142   Santa Ana, CA, USA) following the manufacturers' instructions, and quantified using

143   Qubit®dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). QPCR was

144   performed using primers described in Table 2. The quantification of total bacteria, *Prevotella*

145   *spp., Bacteroides spp.,* Ruminococcaceae, Lachnospiraceae and Negativicutes, *Lactobacillus*

146   *spp.*, enterobacteria and bifidobacteria was performed as described before [24] using a Bio-

147   Rad CFX384 Real Time system and Bio-Rad CFX Manager Software 3.0 (Bio-Rad

148   Laboratories, Watford, UK). DNA concentrations were standardised to 1 ng per well and

149   standard curves consisted of dilution series of amplified bacterial 16S rRNA genes from

150   reference strains as described previously [30]. Samples and standards were run in duplicate and

151   5 ng/µL Herring Sperm DNA (Promega, Southampton, UK) was included in all reactions for

152   stabilization. The efficiencies of standard curves ranged from 92.6 – 104.7% and R^2 values

153   ranged from 0.993 – 0.999 across all primers used.

154

155   *LC-MS/MS analysis*

156   The liquid chromatography analysis of the mycotoxins was performed on an Agilent 1200

157   HPLC system (Agilent Technologies, Wokingham, UK) fitted with an Agilent Zorbax 5 µm,

158   150 mm × 4.6 mm C18 column. The method parameters were described previously [10].

159   Mycotoxins were detected on a Q-Trap 4000 triple quadrupole mass spectrometer (AB Sciex,

160   Warrington, UK) fitted with a Turbo Ion Spray™ (TIS) source. The transitions for DON,

161   DOM-1 and DON3Glc from microbial incubations were: 355.1 → 265.1, 339.1 → 249.1 and

8

162 517.3 → 427.3, respectively. Calibration curves for each metabolite ranged from 0.25 to 2
163 nmol/mL.

164

165 *Statistical analysis*

166 The time course over 72 hours of the DON and DON3Glc hydrolysis results from Figure 1
167 (expressed as % of dose) were used to calculate the area under the curve (AUC, % × hr) for
168 each animal and each intestinal section individually. Bacterial count data were log-
169 transformed to meet requirements of constant variance and normality (based on visual
170 inspection of residual plots). These data were then analysed by ANOVA, with animal as
171 random effect and tissue as fixed effect. When the effect of tissue was significant (P<0.05),
172 tissues were compared by post hoc t-test based on the ANOVA output. The colon sample of
173 one animal was excluded from the statistical analyses due to failure of the qPCR assay. All
174 analyses were carried out using Genstat 17 Release 17.1 (Lawes Agricultural Trust, VSN
175 international Ltd, Hemel Hempstead, UK). A P-value < 0.05 was regarded significant.
176 Results are presented as mean±SEM, based on spread between animals.

177

178 Results
179 This study was conducted to assess the metabolism of DON and DON3Glc by porcine
180 microbiota derived from different regions of the intestinal tract. Results show that
181 detoxification of DON to DOM-1 did not occur in any animal or any gut region (Table 3). No
182 trace of DOM-1 was detectable in any of the samples (data not shown), and recovery of DON
183 ranged from 87-119% of dose following incubation over 24-72 hours.

184   Microbial hydrolysis of DON3Glc was efficient in all pigs and occurred at all intestinal
185 regions tested (Figure 1). In the jejunum, DON3Glc hydrolysis was slowest and free DON was

186   first observed after 24 hours of incubation, increasing to a maximum of 1–41% of the added

187   DON3Glc dose after 72 hours. The ileal microbiota was more efficient in DON3Glc hydrolysis

188   releasing 60±18% of the dose as free DON after 24 hours of incubation. Microbiota of the large

189   intestine hydrolyzed DON3Glc more rapidly with 2 and 3% of the dose detectable as free DON

190   in caecum and colon incubations after 2 hours increasing to 8 and 14% after 6 hours of

191   incubation. Fecal microbiota were most efficient in hydrolyzing DON3Glc with only 4±6% of

192   the dose left as DON3Glc after 9 hours of incubation.

193   The results from the DON3Glc hydrolysis time course experiments (Figure 1) were

194   used to calculate the area under the curve (AUC) for each individual animal and each intestinal

195   region for DON3Glc (Figure 2, top panel) and DON (bottom panel). DON3Glc hydrolysis rates

196   were slowest for all animals in jejunal samples, as indicated by the highest AUC for DON3Glc

197   curves and the lowest AUC for DON curves. Ileal DON3G hydrolysis was significantly faster

198   in all animals (P<0.05) than jejunal hydrolysis, but slower (P<0.05) than rates observed in the

199   large intestine. No differences were observed between DON3Glc hydrolysis rates in caecum,

200   colon and fecal samples.

201   Microbiota composition was analyzed using DNA extracted from untreated digesta

202   samples (without mycotoxin spiking) derived from ileum, caecum, colon and feces of

203   experimental pigs. Total bacterial load showed a tendency (P=0.057) towards differences

204   between intestinal regions, with a lower log count in the ileum compared to the caecum and

205   colon (P<0.05) (Figure 3). Log counts of *Bacteroides* spp., *Prevotella* spp., Ruminococcaceae,

206   Lachnospiraceae and Negativicutes were all lower in the ileum (P<0.05) but did not differ

207   between caecum, colon and feces. In the ileum members of the phylum Firmicutes dominated

208   the microbiota with lactobacilli forming the largest portion of bacteria. However, most bacteria

209   in the ileum were not identified with the primers used, suggesting that the ileum harbors

210   bacteria out with the groups covered here.

211

212   Discussion

213   The current study was conducted to assess the microbial metabolism of the masked mycotoxin

214   DON3Glc and the free form DON by intestinal microbiota derived from different regions of

215   the small and large intestine of pigs. We found no evidence of microbial de-epoxydation of

216   DON to DOM-1 in any digesta sample. Similarly, Eriksen and colleagues found no DOM-1

217   production in ileal or fecal samples from 5 experimental pigs even though DOM-1 production

218   was reported in pigs from commercial farms [14]. Interestingly, 4 of these 5 animals acquired

219   the microbiota capable of DOM-1 production after they were exposed to feces of DOM-1

220   producing animals. This suggests that the microbes capable of DON de-epoxydation are

221   acquired from the environment and confirms that ingestion of DON-contaminated feed may

222   alter the intestinal microbiota [31,32].

223   The study presented here demonstrates that microbiota derived from the porcine small

224   intestine efficiently hydrolyze the masked mycotoxin DON3Glc and release free DON *in vitro*.

225   Furthermore, microbiota from the porcine caecum, colon and feces hydrolyze DON3Glc

226   equally efficiently. Upon ingestion, DON3Glc has been found to be not toxic (in pig intestinal

227   explants [8]) and is not absorbed intact in pigs, but free DON and further metabolites are

228   detectable in plasma and urine. DON3Glc absorption in pigs (as DON) is less efficient

229   compared to free DON (16% vs 81% of dose absorbed after 8 hours [19]) and also slower than

230   DON (42 vs 84% of dose excreted in urine after 24hours [18]). These findings suggest

231   continuous, slow release of DON from DON3Glc prior to absorption, which would be in line

232   with microbial hydrolysis beginning after 6 or 9 hours incubation as reported here.

233   This slow and continuous DON release from DON3Glc exposure may result in

234 toxicities in the more distal regions of the intestine compared to DON dosed animals. There is

235 some evidence that binding of DON to a clay-based feed additive results in DON exerting its

236 intestinal toxicity (disruption of intestinal barrier function, induction of oxidative stress) in the

237 more distal part of the small intestine in chickens when compared to free DON, although

238 colonic tissue was not evaluated in this study [33]. This suggests that binding of DON can lead

239 to the intestinal toxicity being shifted to more distal intestinal regions and it can be

240 hypothesized that plant-bound DON3Glc could act as delivery mechanism to the ileum and

241 colon where microbial hydrolysis will lead to DON exposure and potential toxicity. Upon

242 ingestion of DON3Glc, it would be interesting to determine the absorption and the effect of

243 DON in the large intestine.

244   Microbiota profiling demonstrated that microbiota from the caecum, colon and feces

245 were dominated by *Prevotella* spp., followed by Ruminococcaceae, Lachnospiraceae and

246 Negativicutes. This is in agreement with literature suggesting Bacteroidetes and Fimicutes to

247 be the dominant phyla in the large intestine and feces of pigs [34-36]. Enterobacteria

248 represented a substantial group in the small and large intestine of only one pig, whereas

249 *Bacteroides* spp. and bifidobacteria did not represent major groups in any animal or gut site.

250 This is in contrast with published work [37] reporting *Bacteroides* spp. to be a major group in

251 porcine feces. The current study focused on quantitative and qualitative analysis of the

252 intestinal microbiota of the porcine intestine, but did not identify specific bacterial groups

253 involved in the hydrolysis of DON3Glc. Published work has identified bacteria from very

254 different genera and phyla (lactobacilli, enterococci, bifidobacteria) that are capable of

255 hydrolyzing DON3Glc and other masked mycotoxins [38,39] and future studies are required

256 to understand their contribution to hydrolysis is mixed microbial communities and *in vivo*.

257   The human intestinal microbiota possess several glycosyl hydrolase genes [40] and

258   human fecal microbiota are known to hydrolyse DON3Glc [7,10,11]. It is therefore likely that

259   DON3Glc hydrolysis occurs in the human inestine, but future experiments are required to

260   provide evidence. The fact that the microbial metabolite DOM-1 is present in human urine

261   [10,16,17] further supports the hypothesis that microbial mycotoxin metabolism and absorption

262   occur *in vivo* in humans.

263   In conclusion, the present study demonstrates that masked mycotoxins can contribute

264   to mycotoxin exposure following rapid, efficient and non-specific hydrolysis by intestinal

265   microbiota of the distal small intestine and the large intestine. Potential specific toxicities of

266   microbial mycotoxin release in the distal intestine remain to be investigated in future studies.

267

268   Acknowledgement

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406   Figure legends

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408   Figure 1. Hydrolysis of DON3Glc and release of free DON by porcine intestinal microbiota

409   form different regions of the small and large intestine over 0–72 hours. Results are presented

410   as average of 5 animals±SEM.

411

412   Figure 2. Individual differences in DON3Glc hydrolysis (top panel) and DON release (bottom

413   panel) by intestinal microbiota of 5 animals. Data from time course experiments were

414   summarized by area under the curve (AUC) for each individual animal and gut site. Effect of

415   tissue was significant (P<0.001, ANOVA) for both DON3Glc and DON. Tissues that do not

416   share a superscript are significantly different (P<0.05, post-hoc t-test).

417

418   Figure 3. Microbial community analysis in porcine digesta samples from different regions of

419   the small and large intestine. Results are presented as averages of 5 animals±SEM for ileum,

420   caecum and feces and average of 4 animals±SEM for colon samples. Within those bacterial

421   groups for which the effect of tissue was significant (P<0.05, ANOVA), tissues that do not

422   share a superscript are significantly different (P<0.05).

423

424   Table 1. Summary of feed composition.

Food constituent	Unit	Oligo elements	mg/kg
Raw proteins	17 %	Iron	86
Raw fat	2.5 %	Copper	160
Raw ashes	4.5 %	Manganese	40
Crude fiber	4.5 %	Zinc	110
Phosphorus	0.55 %	Iodine	1
Calcium	0.65 %	Selenium	0.3
Sodium	0.2 %		
Lysine	11.9 g/kg		
Methionine	3.6 g/kg		
Additives	**Units/kg**	**Enzymes**	**Units/kg**
E672 A vitamin	12000	Endo 1, 3 (4) beta glucanase	125 U
E671 D3 vitamin	2000	Endo 1, 4 beta xylanase	87 U
3a700 E vitamin E	60	Phytase	1880 U

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429   Table 2. Summary of group-specific qPCR primers.

Target group	Sequence	T	Amplicon	Reference strain	Ref.
Universal	GTGSTGCAYGGYYGTCGTCA	60	141	*Ruminococcus bromii* L2-63	[23]
	ACGTCRTCCMCNCCTTCCTC				
Prevotella spp.	CRCRCRGTAAACGATGGATG	65	105	*Prevotella copri* DSM18205	[24]
	TTGAGTTTCACCGTTGCCGG				
Bacteroides spp.	GCTCAACCKTAAAATTGCAGTTG	63	110	*Bacteroides thetaiotamicron* B5482	[24]
	GCAATCGGRGTTCTTCGTG				
Lactobacillus spp.	AGCAGTAGGGAATCTTCCA	60	341	*Lactobacillus reuteri* DSM20016	[25]
	CACCGCTACACATGGAG				[26]
Bifidobacteria	TCGCGTCYGGTGTGAAAG	60	128	*Bifidobacterium adolescentis* DSM20083	[23]
	GGTGTTCTTCCCGATATCTACA				
Enterobacteria	GACCTCGCGAGAGCA	60	180	*Escherichia coli* XL1Blue	[27]
	CCTACTTCTTTTGCAACCCA				
Cluster IV Ruminococcaceae family	GCACAAGCAGTGGAGT[1]	60	241	*R. bromii* L2-63	[28]
	GCACAAGCGGTGGATT[1]				
	CTTCCTCCGTTTTGTCAA				
Cluster IX Negativicutes class	GTTGTCCGGAATYATTGGGC	63	321	*Megasphaera elsdenii* LC1	[29]
	ATTGCGTTAACTCCGGCACA[2]				
	ATTGCGTTAACTCCGGCACG[2]				
Cluster XIVa Lachnospiraceae family	CGGTACCTGACTAAGAAGC	60	429	*Roseburia hominis* A2-183	[30]
	AGTTTYATTCTTGCGAACG				

430   Both primers ([1]forward primers for cluster IV, [2]reverse primers for cluster IX primers) were
431   used together at equimolar concentration. T Annealing temperature.
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437   Table 3. Recovery (% of dose 2 nmol/mL) of DON from microbial incubations.

Time	Jejunum	Ileum	Caecum	Colon	Feces
0 hr	100.3 (±0.3)	102.7 (±1.2)	98.6 (±1.1)	100.5 (±0.3)	99.9 (±0.1)
24 hr	103.9 (±6.7)	97.2 (±8.4)	94.0 (±9.3)	99.6 (±8.3)	119.4 (±5.7)
48 hr	87.3 (±4.3)	90.0 (±6.1)	113.9 (±6.2)	103.9 (±9.3)	114.6 (±8.7)
72 hr	90.3 (±6.2)	91.8 (±6.6)	116.0 (±6.5)	108.1 (±9.0)	113.6 (±7.8)

438   Results presented as average of 5 animals±SEM.