

High-dose cannabidiol induced hypotension after global hypoxia-ischemia in piglets.

Running title: CBD after neonatal hypoxia-ischemia

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Abstract

Background: Cannabidiol (CBD) is considered a promising neuroprotectant in the setting of perinatal hypoxia-ischemia (HI). We have previously studied the effects of CBD 1 mg/kg in the early phase after global HI in piglets. In contrast to existing studies found no evidence of neuroprotection and hypothesized that higher doses might be required to demonstrate efficacy in this animal model.

Objective: To assess the safety and possible neuroprotective effects of high-dose CBD.

Methods: Anesthetized newborn piglets underwent global HI by ventilation with 8% O₂ until the point of severe metabolic acidosis (BE \leq -20 mmol/l) and/or hypotension (MABP \leq 20 mmHg). Piglets were randomized to intravenous treatment with vehicle (n=9) or CBD (n=13). The starting dose of CBD 50 mg/kg was reduced if adverse effects occurred. Piglets were euthanized 9.5 hours after HI and tissue was collected for analysis.

Results: CBD 50 mg/kg (n=4) induced significant hypotension in 2 out of 4 piglets and 1 out of 4 piglets suffered fatal cardiac arrest. CBD 25 mg/kg (n=4) induced significant hypotension in 1 out of 4 piglets, while 10 mg/kg (n=5) was well tolerated. A significant negative correlation between the plasma concentration of CBD and hypotension during drug infusion was observed ($p < 0.005$). Neuroprotective effects were evaluated in piglets that did not display significant hypotension (n=9) and CBD did not alter the degree of neuronal damage as measured by neuropathology score, the levels of the astrocytic marker S100B in CSF, magnetic resonance spectroscopy markers (Lac/NAA & Glu/NAA ratios) or troponin-T in plasma.

Conclusions: High-dose CBD induced severe hypotension and did not offer neuroprotection in the early phase after global HI in piglets.

Introduction

Cannabidiol (CBD), a non-psychoactive cannabinoid, has over the last decade emerged as a promising neuroprotectant for the treatment of neonatal hypoxic-ischemic encephalopathy [1-6] and has received orphan designation status for this condition [7].

Neuroprotection by CBD after perinatal hypoxia-ischemia (HI) has been demonstrated after doses ranging from 0.1-1000 μ M in whole brain-slices from mice [8], 1-30 mg/kg intraperitoneally in rats [9] and 0.1-1 mg/kg intravenously (i.v) in piglets [3-5]. We recently studied the effects of CBD 1 mg/kg i.v in a piglet model of global hypoxia-ischemia (HI) [10] and in contrast to previous studies in piglets [2-5] no neuroprotective effects were found early phase after HI. The hypoxic-ischemic insult in our model produces a more severe multi-organ damage compared to these studies [2-5]. As the effects of cannabinoids have been shown to depend both on the dose and pathophysiological setting [11,12] we hypothesized that higher doses of CBD could be required to demonstrate efficacy in this animal model.

However, when considering high-dose CBD in this setting it is important to be aware of its possible effects on other organs. Like the cardiovascular system where CBD's actions on the heart and vasculature have been demonstrated in several in-vitro studies [12-15]. Even though intravenous CBD up to 100 mg/kg in animal models [16] and 30 mg/kg in humans [16,17] have been well tolerated, these studies did not

involve global HI which is known to induce cardiovascular dysfunction [18]. In piglets exposed to HI the highest dose of CBD yet to be tested is 1 mg/kg i.v and it did not produce unwanted cardiovascular effects [3,4,10].

Hence the aim of this study was to evaluate the safety of high-dose CBD (>10 mg/kg) after global HI in piglets, and further to perform a preliminary assessment of possible neuroprotective effects.

Materials and methods

Approval

The Norwegian Council for Animal Research approved the experimental protocol (approval number 7359). The animals were cared for and handled in accordance with the European Guidelines for Use of Experimental Animals, by certified FELASA (Federation of European Laboratory Animals Science Associations) Category C researchers.

Experimental protocol

The animal model used have been extensively described previously [10]. Briefly; 22 newborn Noroc (LyxLD) pigs were exposed to hypoxia-ischemia (HI) by ventilation with a gas mixture of 8% O₂ in N₂ until either the mean arterial blood pressure (MABP) decreased to 20 mmHg or the base excess (BE) reached -20 mmol/L. After resuscitation piglets were randomized, by sealed envelopes, to treatment with high-

dose CBD (n=13) or Vehicle (VEH) (n=9). We have previously demonstrated a significant effect of HI on the parameters assessed in this study [10] and thus a non-hypoxic control group was not included in the current experiments.

The MABP was measured continuously in the left carotid artery using BioPac systems MP150-CE (Goleta, USA). The O₂ saturation was monitored by pulseoxymetry (Masimo RAD-5 pulseoxymeter, Neuchatel, Switzerland). Rectal temperature was measured by digital rectal thermometer and maintained between 38.5 and 40.0°C. Temperature-corrected arterial acid/base status, glucose and hemoglobin were regularly measured on a Blood Gas Analyzer 860 (Ciba Corning Diagnostics, Midfield, Mass., USA). Apart from during the hypoxic insult mechanical ventilation settings were adjusted to maintain normoventilation.

Blood and CSF for analysis were obtained at the end of the experiment (9.5 hours after HI). Samples were immediately snap-frozen in liquid nitrogen and stored at minus 80°C. The animals were euthanized with an overdose of pentobarbital (150 mg kg⁻¹ IV). The brain was immediately removed and sagittally divided. From the right half, specimens from the fronto-parietal cortex and the hippocampus were snap-frozen in liquid nitrogen and stored at -80°C until subsequent analysis. The left hemisphere was placed in 4% buffered formalin for subsequent histopathological analysis.

Dose and administration of cannabidiol

A starting dose of CBD 50 mg/kg (GW Research Ltd, Cambridge, UK) was chosen based on existing literature [16]. If significant side effects occurred the dose was to

be reduced step-wise to 25 mg/kg, 10 mg/kg and further to 5 mg/kg. Side effects evaluated were severe hypotension, defined as drop in blood pressure to < 70% of baseline MABP (start infusion).

CBD was prepared in a 10mg/ml formulation of ethanol:solutol:saline at a ratio of 2:1:17. CBD was mixed with saline to give a total volume of 20 ml. Vehicle treated animals received an equivalent amount of vehicle to the CBD 50 mg/kg dose regimen. CBD and vehicle were given 30 minutes after the end of hypoxia i.v over 15 minutes.

Plasma and brain concentrations CBD

Plasma and brain levels of CBD were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at LGC (Fordham, UK).

Histology

A transcoronal block (~ 0,4 cm thick) from the level of parietal cortex including basal ganglia was prepared, processed in a Leica TP1050 tissue processor (GMI, Minnesota, 55303, USA), embedded in paraffin, sliced in 4- μ m-thick sections and stained with hematoxylin and eosin (H&E).

Analysis was performed by an experienced pathologist blinded to the randomization/clinical details. Areas with vacuolated neuropil, shrunken neurons with pyknotic nuclei and scattered eosinophilic neurons were defined as early neuronal damage. A modified version of a validated scoring system [19] was used. Based on evaluation of the whole brain section, including cortex, white matter and

basal ganglia, a global score with 8 levels, ranging from 0-4, was given to each piglet. Zero representing no damage, 0.5 – 1.5: mild/moderate, 2-2.5: moderate/severe, 3-3.5: severe and 4: massive damage with autolysis of the brain.

Biomarkers

S100B in CSF and plasma Troponin-T were measured using electrochemiluminescent immunometric assay (ECLIA) on the Cobas e601 immunoassay platform (Roche D, Mannheim, Germany).

Proton-magnetic-resonance-spectroscopy (H⁺-MRS)

H⁺-MRS was performed by the collaborative unit in Madrid in the MRI Unit of the Instituto Pluridisciplinar (Universidad Complutense, Madrid, Spain) and the method is extensively described in the papers by Pazos et al [4] and Lafuente et al [3].

Statistical analysis

Statistical analyses were performed by SPSS v.21 (Chicago, USA) and GraphPad Prism 6 (GraphPad Prism Software Inc, La Jolla, CA). Non-normally distributed data were log transformed and if unsuccessful non-parametric statistics was used. Comparison of cohort characteristics were analyzed by one-Way ANOVA with Dunn`s post-hoc test. Descriptive results are presented with means \pm standard deviations, standard error of mean or median with intra-quartile range as appropriate. High-dose CBD was compared to VEH by independent t-tests or Mann-whitney test. Correlations were calculated by Spearman rank correlation coefficient. A 5% significance level was used.

Results

Cohort description

Twenty-two piglets were included in the study (VEH=9 and CBD=13). No significant differences in baseline physical variables or biophysical and biochemical variables were observed between the study groups (table 1)

Plasma and brain concentrations

The plasma and brain concentrations of CBD showed a linear relationship with the dose (figure 1). CBD levels in the brain were strongly correlated with plasma levels ($\beta = 0.4 \mu\text{g}$, 95% CI [0.3 to 0.5], $p < 0.0001$) (fig 1 c).

Safety

There were no significant group differences in hemodynamic variables before treatment (table 1). No significant difference in heart rate was observed during treatment (table 1). 3/4 piglets in the CBD 50 mg/kg group displayed significant hypotension, with a drop in MABP below 70% of baseline. One of these piglets suffered a fatal cardiac arrest immediately after the end of infusion. The mean drop was significant compared to VEH (CBD - 26 ± 9 mmHg vs. VEH - 2 ± 4 mmHg, $p < 0.0001$) (fig 2 & 3a). 1/4 piglets receiving 25 mg/kg displayed severe hypotension, but group mean drop was not significant (CBD 25 mg/kg - 8 ± 10 mmHg vs. VEH - 2 ± 4 mmHg, $p = 0.29$). VEH and CBD 10 mg/kg did not produce significant hypotension. We observed a significant negative association between the plasma concentration of

CBD at 3 hours post-dose and MABP with a mean drop of -1 mmHg (95% CI [-1.7 to -0.5], $p = 0.002$) per 1 μg increase in CBD (fig 3b)).

Neuroprotection

The neuropathology score was not significantly different in piglets receiving high-dose CBD compared to VEH (CBD score 1.9 ± 1.4 vs. VEH 1.6 ± 1.3 , $p=0.67$) and CBD did not significantly alter the levels of S100B in cerebrospinal fluid (CBD 17.8 ± 11.9 ng/ml vs. VEH 11.5 ± 11.9 ng/ml, $p=0.3$). High-dose CBD did not mitigate the increase in Lac/NAA ratio (CBD 3.8 (3.4- 9.9) vs. VEH 3.7 (3.3-4.9), $p=0.55$) or Glu/NAA ratio (CBD 1.4 ± 0.3 vs. VEH 1.3 ± 0.2 , $p=0.44$). Levels of plasma troponin-T were not significantly different in piglets who received high-dose CBD compared to VEH (CBD 155 ± 179 ng/L vs. VEH 240 ± 230 ng/L, $p=0.42$) (results summarized in table 2).

The above analysis included all animals receiving high-dose CBD ($n=9$) with vehicle ($n=9$), excluding piglets who displayed severe hypotension during treatment ($n=4$). Sub-group analysis of the individual doses of CBD had, prior to this, not shown significant differences among groups.

Discussion

In this study we have evaluated the safety of high dose CBD in newborn piglets exposed to global HI as well as performed a preliminary assessment of efficacy. We

have demonstrated that high-dose CBD in this setting can induce severe hypotension in some animals. Further, high-dose CBD did not offer neuroprotection in the first 9.5 hours after HI as evaluated by a neuropathology score, levels of the astrocytic marker S100B in cerebrospinal fluid, hippocampal proton magnetic resonance spectroscopy biomarkers or plasma troponin-T levels.

CBD has been shown to induce hypotension in animals previously [12], but in most studies even high doses has been well tolerated [16]. However, these models did not induce severe global HI. Cardiovascular dysfunction in the current animal model is demonstrated by severe hypotension during hypoxia-ischemia and in the recovery phase; a degree of persistent hypotension and tachycardia as well as significantly elevated plasma troponin-T [10]. High-dose CBD did not induce cardiac arrhythmias or significant alterations in heart rate. Still, in this setting of a compromised myocardial function [18], CBD's vasorelaxant effects [14,20], its ability to depress the contractility of myocytes [15], and to reduce the cardiovascular response to stress [13] can all be potential explanations for the observed hypotension.

A significant correlation between the plasma concentrations of CBD and the degree of hypotension during infusion was observed. All piglets with a significant drop in MABP had plasma levels of CBD above 4 micrograms/ml at 3 hours post dose. This corresponds to an estimated molar concentration of approximately 13 μ M. The initial concentrations, however, are likely far greater as C_{max} after intravenous

administration is achieved almost instantaneously. In an in-vitro study on the pre-constricted rat aorta, CBD 5 to 10 μM were found to induce a 25-50 % relaxation respectively [20] and in another in-vitro study on rat myocytes, CBD as low as 1 μM were found to exert negative inotropic effects [15]. We should, however be cautious when extrapolating the effects of in-vitro concentrations to in-vivo studies [21] as well as across species. Modifications in the method of CBD administration, e.g. by using a slower infusion rate or giving CBD later when cardiovascular function has improved, could have limited the observed effects. Further, we did not treat hypotension with vasopressors or inotropic drugs. Nonetheless, the possibility of inducing unwanted hypotension is a possible limitation of the highest doses of CBD in this setting.

We found no significant protective effects of high-dose CBD on the levels of early neuronal damage assessed by neuropathology. Nor did CBD alter the levels of S100B in cerebrospinal fluid or plasma troponin-T, both of which have been found to predict outcome after perinatal HI [18,22]. Proton magnetic spectroscopy biomarkers, especially the Lac/NAA ratio are early predictors of outcome after neonatal HI [23], but were not influenced by high-dose CBD in our study. Although we have evaluated a limited number of variables, they have all been used previously to demonstrate CBD's neuroprotective effects in piglets [3-5]. Explanations for the lack of effects in our model compared to previous studies in piglets [2-5] have been thoroughly discussed before [10]. Most relevant is probably the difference in the

nature of HI. Among others, the duration of HI is longer in our model (40-55 vs. 20-30 minutes) and piglets have a more profound metabolic acidosis (pH 6.85 vs. 7.2).

In regard to neuroprotection; an important limitation of this study is the early end-point used for analysis as it allows us only to study effects limited to the latent phase and early secondary energy failure after perinatal HI [24]. Nevertheless the majority of the existing literature in support of CBD's neuroprotective effects after neonatal HI, have used comparable end-points (6 hours) [2-4]. As such the evidence of CBD's full potential after perinatal HI is still limited and difficult to interpret. Further, we acknowledge the relatively small sample size and the variation in degree of injury after HI seen in this model. The animals excluded due to severe hypotension did not display significant differences in the outcome variables related to neuroprotection and including them would not have altered the overall results. Nevertheless, we found it most appropriate to exclude them based on the probability of brain ischemia caused by such a severe drop in MABP, which is considered to be below the lower limit of cerebral auto-regulation in piglets [25].

Before considering clinical trials with CBD in the setting of perinatal HI it is important to confirm its safety and efficacy in different preclinical models and in our opinion this study has relevance in this regard. Although adverse effects were seen only after the highest doses, and might be prevented by modifications in the timing and method of administration, they are important to consider in future studies. In the

current animal model CBD has failed to demonstrate neuroprotective effects, yet its full neuroprotective potential and optimal dose remains to be determined.

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Tables

	Vehicle (n=9)		CBD 10mg/kg (n=5)		CBD 25 mg/kg (n=4)		CBD 50 mg/kg (n=4)*		P
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Weight (grams)	2008	86	1900	121	1953	115	2069	90	ns
Age (hours)	28	1	26	2.9	29	4	28	2	ns
Hb (g/dl)	6.6	0.8	6.4	1.0	7.1	0.7	6.6	2.0	ns
Hypoxia (min)	62	30	57	20	54	28	59	23	ns
Tp (C°)									
S	39.1	0.5	38.5	1.3	38.5	0.8	39.5	0.7	ns
E	38.2	0.6	38.7	0.6	37.9	0.5	38.3	0.5	ns
T	38.7	0.6	38.4	0.7	38.0	1.0	38.4	0.8	ns
180	39.0	0.9	38.9	0.6	38.8	0.3	38.5	0.6	ns
ES	38.6	0.8	38.6	0.5	38.8	0.5	38.8	0.6	ns
MABP (mmHg)									
S	60.0	7.3	57.0	9.7	57.0	4.5	60.0	2.5	ns
E	33.0	16.7	27.0	7.9	32.0	12.5	32.0	9.9	ns
T	48.0	10.5	44.0	4.4	53.0	7.3	50.0	6.2	ns
HR (bpm)									
S	154	12	156	21	130	27	141	31	ns
E	181	30	160	26	172	19	189	24	ns
T	197	32	210	11	180	28	196	31	ns
ET	196	31	222	19	187	22	166	58	ns
pH									
S	7.4	0.1	7.4	0.1	7.4	0.1	7.4	0.1	ns
E	6.9	0.1	6.9	0.1	6.9	0.1	6.9	0.0	ns
30	7.2	0.1	7.2	0.1	7.2	0.1	7.2	0.0	ns
180	7.4	0.1	7.4	0.1	7.4	0.1	7.4	0.1	ns
ES	7.4	0.1	7.4	0.0	7.4	0.0	7.4	0.0	ns

Lactate (mmol/l)									
S	1.8	0.6	1.5	0.8	1.6	0.8	3.6	2.1	ns
E	14.5	2.2	12.9	1.3	15.6	3.0	13.9	2.4	ns
T	12.2	2.4	11.3	2.1	11.9	2.4	11.7	3.2	ns
180	2.3	1.3	1.7	0.6	1.4	0.3	2.7	1.8	ns
ES	1.5	1.4	1.1	0.3	1.0	0.3	0.8	0.2	ns
BE (mmol/l)									
S	3.0	3.1	1.7	1.7	1.9	1.6	-1.0	2.7	ns
E	-18.1	3.0	-18.7	2.2	-18.1	2.4	-18.6	0.9	ns
T	-13.0	1.7	-13.6	2.5	-12.5	3.7	-13.1	1.6	ns
180	1.1	2.1	-0.2	1.4	2.8	2.8	-1.3	1.3	ns
ES	-0.6	3.2	-0.5	1.5	1.0	2.6	0.2	1.3	ns

Table 1: Cohort description. Biophysical and biochemical variables at start HI (S), end HI (E), start of treatment (CBD/VEH) (T), end of treatment (ET), 180 min post treatment (180) and end of study, 540 min post treatment (ES).

Hb= hemoglobin, Tp= temperature, MABP=mean arterial blood pressure, HR= heart rate. BE= base excess ns = non significant. Statistics by one-way Anova with Dunnet`s post hoc test. *One piglet in this group suffered fatal cardiac arrest immediately after CBD infusion and n is therefore 3 at 180 min post HI and at end-study.

	Vehicle (n=9)	High-dose CBD (n=9)	P-value
Neuropathology score	1.6 (1.3)	1.9 (1.4)	0.67
S100B ng/ml	11.5 (11.9)	17.8 (13.1)	0.30
Lac/NAA ratio*	3.7 (3.3 - 4.9)	3.8 (3.4 - 9.9)	0.55*
Glu/NAA ratio	1.3 (0.2)	1.4 (0.3)	0.44
Troponin-T ng/L	240 (230)	155 (179)	0.42

Table 2: Neuroprotection. Comparison of the effects of high-dose CBD (excluding those with severe hypotension following infusion) vs. vehicle on the different outcome variables related to neuroprotection. Data are presented as mean (standard deviation) or median with intra-quartile range (*). Statistics was performed by independent t-test or Mann-Whitney test (*).

Figures

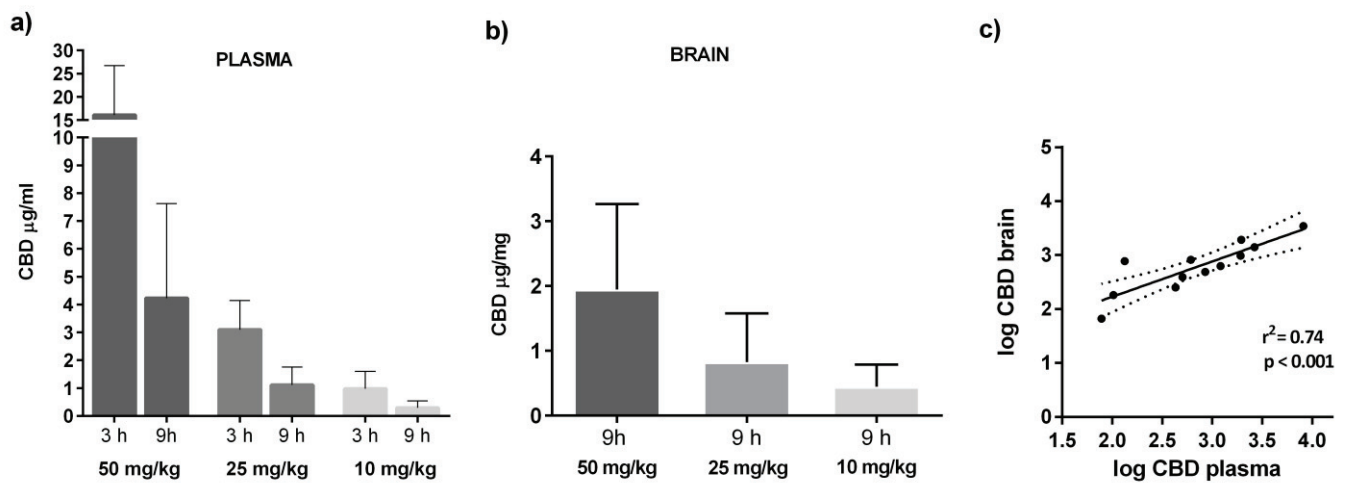


Fig 1: Plasma and brain concentration of CBD. Concentrations of CBD in plasma at 3 and 9 hours post-treatment (a) and brain 9 hours post-treatment (b). Correlation between plasma and brain levels at 9 hours post-treatment(c). Data are presented as mean \pm SD (a-b).

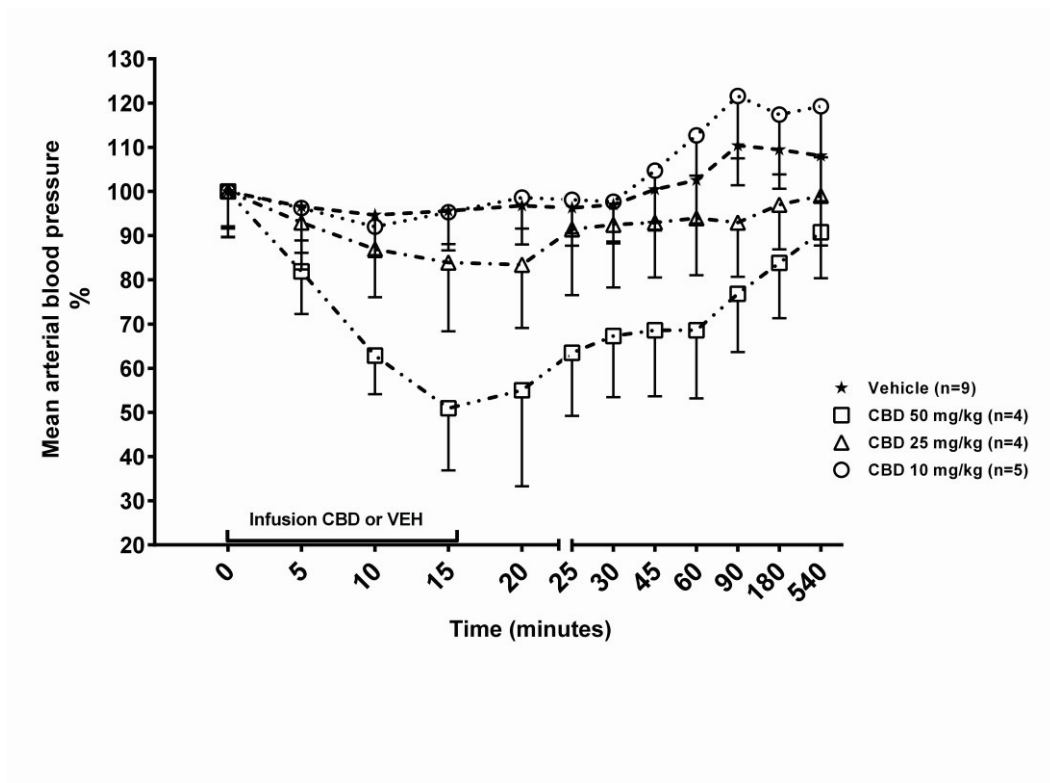


Fig 2. Blood pressure changes. Relative changes in mean arterial blood pressure (MABP) during infusion of CBD or VEH. Data presented as mean (-SEM) VEH: cross, CBD 50 mg/kg (squares) CBD 25 mg/kg (triangles) CBD 10 mg/kg (circles). Note that one piglet receiving CBD 50 mg/kg suffered fatal cardiac arrest immediately after end of infusion and thus n=3 in this group from 20 – 540 minutes.

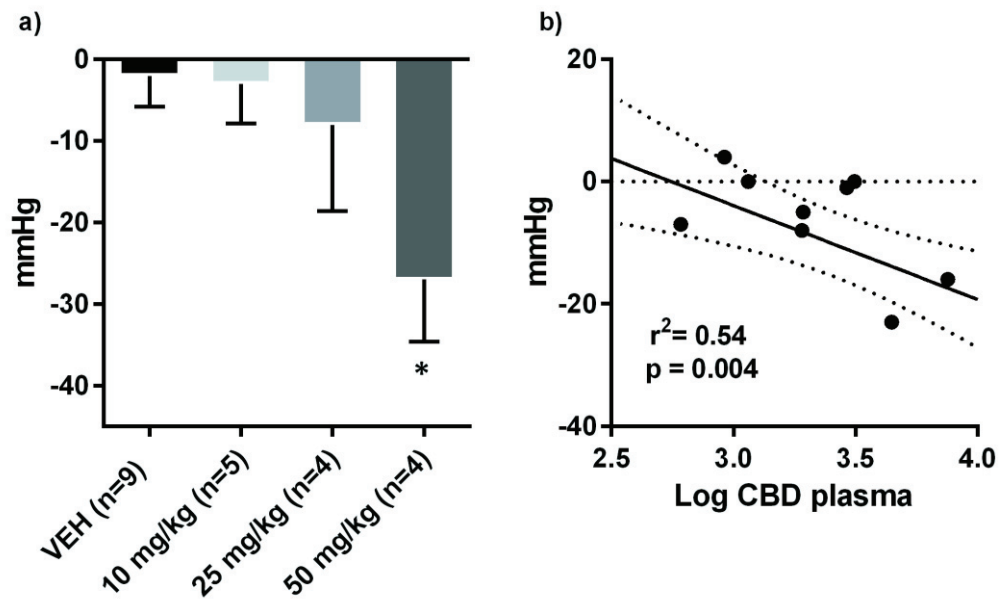


Fig 3: Blood pressure changes 2. Mean drop in MABP per group (a). Correlation of plasma concentrations of CBD 3 h post-treatment with the degree of hypotension during infusion (b). Data presented as mean \pm SD. Statistics by One-way ANOVA with the Dunnett's post-hoc test * $p < 0.0001$.