1   Involvement of monocyte-derived extracellular vesicle-associated tissue factor activity in

2   convallatoxin-induced hypercoagulability

3

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14   **Short Title:** EV-associated TF in convallatoxin poisoning

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23

27 **Abstract**

28 **Objectives:** Convallatoxin (CNT) is a natural cardiac glycoside extracted from lily of the valley

29 (*Convallaria majalis*). Although it is empirically known to cause blood coagulation disorders, the

30 underlying mechanism remains unclear. CNT exerts cytotoxicity and increases tissue factor (TF)

31 expression in endothelial cells. However, the direct action of CNT on blood coagulation remains

32 unclear. Therefore, herein, we investigated the effects of CNT on whole blood coagulation

33 system and TF expression in monocytes.

34 **Methods:** Blood samples were collected from healthy volunteers to measure plasma

35 thrombin-antithrombin complex (TAT) concentration using ELISA and to perform rotational

36 thromboelastometry (ROTUM) and whole-blood extracellular vesicle (EV)-associated TF (EV-TF)

37 analysis. The effects of CNT were also investigated using the monocytic human cell line THP-1.

38 Quantitative real-time PCR and western blotting were performed, and PD98059, a MAPK

39 inhibitor, was used to elucidate the action mechanism of CNT-mediated TF production.

40 **Results:** CNT treatment increased EV-TF activity, shortened the whole blood clotting time in

41 ROTEM analysis, and increased TAT levels, which is an index of thrombin generation.

42 Furthermore, CNT increased *TF* mRNA expression in THP-1 cells and EV-TF activity in the cell

43 culture supernatant. Therefore, CNT may induce a hypercoagulable state with thrombin

44 generation, in which elevated EV-TF activity derived from monocytes might be involved. These

45   procoagulant effects of CNT were reversed by PD98059, suggesting that CNT-induced TF

46   production in monocytes might be mediated by the MAPK pathway.

47   **Conclusions:** The findings of the present study have further clarified the procoagulant

48   properties of CNT.

49

50   **Keywords:** convallatoxin, procoagulant, tissue factor, monocyte, anticancer, cardiac glycoside.

51   **Introduction**

52   The lily of the valley (*Convallaria majalis*) exerts toxic effects on both humans and animals [1,2].

53   The symptoms of lily of the valley toxicity are diverse. The lily of the valley contains an active

54   cardiac glycoside, convallatoxin (CNT), which exerts digitalis-like toxicity and can cause

55   headache, vomiting, dizziness, cardiovascular symptoms, such as arrhythmia and hypotension,

56   and even death. In addition, it is empirically known that the toxic components of lily of the valley

57   have blood-clotting effects [3]. However, the mechanism by which CNT affects blood clotting

58   remains unknown.

59   In previous studies, we detected CNT-induced tissue factor (TF) expression in

60   endothelial cells, and found that this endothelial cell-derived TF may trigger a hypercoagulable

61   state in CNT poisoning [4]. However, the mechanism of direct action of CNT on blood

62   components remains unclear. Recently, the importance of the prothrombotic properties of

63   extracellular vesicles (EVs) has become widely recognized [5]. According to the cell-based

64   theory of coagulation, the first factor involved in the coagulation cascade is TF, which binds to

65   activated factor VII in the bloodstream and evokes the extrinsic system, thereby inducing the

66   production thrombin albeit a small amount and accounting for the initiation phase of coagulation

67   [6]. Activated monocytes release EVs that contain TF on their surface [7]. Therefore, we focused

68   on monocytes among the various blood components because monocytes represent one of the

69   most important physiological expression sites of TF [8]. In the present study, we aimed to assess

70   the procoagulant properties of CNT using whole-blood rotational thromboelastometry (ROTEM)

71   and the involvement of monocytes in CNT-induced coagulation disorders with respect to TF.

72

73   **Methods**

74   *Blood sampling*

75   Blood samples were obtained via venipuncture into 3.8% sodium citrate (9:1, v/v)-containing

76   tubes from healthy volunteers aged 20–50 years who had not taken any medication for 2 weeks.

77   The initial 3 mL of the withdrawn blood was discarded. Written informed consent was obtained

78   from all participants. The study was conducted in accordance with the Declaration of Helsinki

79   and all experimental procedures were approved by the Nara Medical University Ethical Review

80   Committee (approval no. 2071).

81

82   *Cell culture*

83   We used the monocytic human cell line THP-1 (European Collection of Authenticated Cell

84   Cultures [ECACC], Public Health England, Salisbury, UK) as a substitute for monocytes, which

85   are major producers of TF among blood components [8]. THP-1 cells were cultured in Roswell

86   Park Memorial Institute-1640 medium (FUJIFILM Wako Pure Chemicals, Osaka, Japan). Before

87   each assay, THP-1 cells were serum-starved in 0.1% BSA-containing medium for 2 h. For

88   stimulation, CNT was used at 100 nM (final concentration [f.c.]). Phosphate-buffered saline

89　(PBS; FUJIFILM Wako Pure Chemicals) was used as a control. In few experiments, PD98059

90　(Cell Signaling Technology, Danvers, MA, USA), which specifically inhibits the MEK-1-mediated

91　activation of mitogen-activated protein kinase (MAPK), was added to the culture medium (10 µM

92　f.c.).

93　*EV-associated TF activity (EV-TF activity)*

94　EV-TF activity was measured using a two-stage clotting assay, as previously described [7,9]. In

95　the whole-blood EV-TF analysis, CNT (Sigma–Aldrich, St. Louis, MO, USA) was added to

96　citrated human whole blood at f.c. of 100 nM, 1,000 nM, and 10,000 nM to determine the optimal

97　concentration. PBS was used as a control (0 nM f.c.). Plasma samples were obtained after 4 h of

98　stimulation with CNT via centrifugation at 2000 × g for 10 min at room temperature (25–28 °C).

99　To measure EV-TF activity in the culture media of THP-1 cells, 100 nM (f.c.) CNT or PBS was

100　added to the media. After 4 h of stimulation with CNT, the plasma or the culture medium was

101　centrifuged twice at 20 000 × g for 15 min at 4 °C to obtain the EV pellet. The EV pellet was

102　resuspended in 160 µL of HEPES buffer saline containing bovine serum albumin (HBSA; pH 7.4,

103　137 mmol/L NaCl, 5.38 mmol/L KCl, 5.55 mmol/L glucose, 10 mmol/L HEPES, and 0.1% BSA).

104　The resuspended EV samples were placed in a 96-well microplate (40 µL/well). The samples

105　were then incubated for 15 min with 11 µL of an inhibitory mouse anti-human TF antibody

106　(HTF-1; Abcam, Cambridge, UK; Product No. ab234267) (36.4 µg/mL [7.8 µg/mL f.c.]) or 11 µL of

107　a control mouse IgG (Sigma–Aldrich). Next, 50 µL of a mixture containing human FVIIa (4.8

108   nmol/L [2.4 nmol/L f.c.]; Enzyme Research Laboratories, South Bend, IN, USA) and human FX

109   (146.4 nmol/L [73.2 nmol/L f.c.]; Enzyme Research Laboratories) in HBSA containing 10 mmol/L

110   (5 mmol/L f.c.) calcium chloride (pH 7.4) was added. After 2 h of incubation, FXa generation was

111   terminated by addition of 25 μL of HBSA with 25 mmol/L EDTA (pH 7.4) (5 mmol/L f.c.).

112   Subsequently, 25 μL of chromogenic substrate (RGR-XaChrom; 4 mmol/L [0.67 mmol/L f.c.],

113   Enzyme Research Laboratories) was added. The absorbance at 405 nm was measured using a

114   microplate reader (SpectraMax M2; Molecular Device, San Jose, CA, USA). Recombinant TF

115   Innovin (DADE Behring, Marburg, Germany) was used as a standard.

116

117   *ROTEM*

118   ROTEM (Pentapharm, Munich, Germany) measures the global viscoelastic properties of

119   whole-blood clot formation under low-shear conditions [10]. The following numerical parameters

120   were obtained: clotting time (CT), which is defined as the time from the start of the clotting

121   reaction to initial fibrin formation and clot formation time (CFT) is a measure of the rate at which a

122   clot forms with a certain viscoelastic strength. CT plus CFT is a useful indicator of whole-blood

123   clotting, as previously described [11]. To evaluate the effects of CNT on whole-blood clotting,

124   CNT (100 nM f.c.) was mixed with normal whole blood and incubated at 37 °C for 4 h. PBS alone

125   was used as a negative control. After incubation, 300 μL of whole blood was dispensed into the

126   cuvettes and ROTEM was started in NATEM mode by adding 20 μL of 0.2 mol/L CaCl$_2$.

127

128 *Thrombin-antithrombin complex (TAT) enzyme-linked immunosorbent assay (ELISA)*

129 CNT (100 nM f.c.) or PBS (control) was added to citrated whole blood. After 4 h of incubation at

130 37 °C, the whole blood sample was centrifuged at 2000 × g for 15 min to obtain plasma samples.

131 Plasma TAT concentration was measured using a TAT ELISA kit (Novus Biologicals, Centennial,

132 CO, USA) according to the manufacturer's instructions.

133

134 *Quantitative real-time polymerase chain reaction*

135 Total RNA was extracted from THP-1 cells using an RNeasy Mini Kit (Qiagen, Valencia, CA,

136 USA). Total RNA (1 µg) was reverse-transcribed into cDNA using the SuperScript IV RT kit (Life

137 Technologies, Carlsbad, CA, USA), and real-time polymerase chain reaction analysis was

138 performed on a StepOne system (Applied Biosystems, Foster City, CA, USA) using TaqMan

139 probes and primers for human *TF* (assay ID; Hs01076032_m1). Human hypoxanthine

140 phosphoribosyltransferase 1 (*HPRT1*, assay ID Hs02800695_m1) was used as an endogenous

141 control.

142

143 *Western blot analysis*

144 After serum starvation, THP-1 cells were pre-incubated with or without PD98059 (10 µM f.c.) for

145 1 h. CNT (100 nM f.c.) was then added to the culture medium. After 1 h stimulation, THP-1 cells

146   were lysed using T-PER Tissue Protein Extraction Reagent containing protease and

147   phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). Protein concentrations

148   were determined using the bicinchoninic acid assay (Thermo Fisher Scientific), and 10 µg of

149   protein samples were used for western blotting after sodium dodecyl sulfate–polyacrylamide gel

150   electrophoresis. Rabbit monoclonal phosphor-p44/42 MAPK antibody (1:2000, Cell Signaling

151   Technology, #4370) was used to detect signal transduction based on MAPK phosphorylation.

152

153   *Statistical analysis*

154   Data were analyzed using the SPSS software version 19 (IBM, Armonk, NY, USA).

155   Multiple-group comparisons were performed using the Tukey–Kramer test. Student's t-test was

156   used for two-group comparisons (ROTEM and TAT analyses). Differences were assessed at a

157   significance level of $p < 0.05$. Data are presented as the mean ± standard deviation.

158

159   **Results**

160   *CNT treatment induces EV-TF activity in whole blood in a dose-dependent manner*

161   We first assessed EV-TF activity to confirm the appearance of TF-bound EVs in whole blood in

162   the presence of CNT. As expected, EV-TF activity was enhanced in the presence of CNT in a

163   dose-dependent manner (Fig. 1). As 100 nM (f.c.) of CNT was sufficient to induce EV-TF activity,

164   a f.c. of 100 nM was used in subsequent experiments.

165

166   *CNT treatment enhances whole-blood coagulation*

167   Next, to confirm the procoagulant activity of CNT in whole blood, ROTEM was performed. As

168   withdrawn blood was used for the experiments, the effects of endothelial cells on blood

169   coagulation can be excluded. In the ROTEM traces, CT plus CFT appeared to shorten in the

170   presence of CNT (Fig. 2a). CT plus CFT was significantly reduced in the presence of CNT

171   compared with that in the absence of CNT ($p < 0.05$, Fig. 2b).

172

173   *CNT treatment increases TAT levels in blood plasma*

174   TAT levels in the blood plasma increased significantly after incubation of whole blood with CNT

175   (100 nM f.c.) ($p < 0.05$, Fig. 3). Given that TAT is a surrogate marker for thrombin generation [12],

176   the elevation of TAT levels indicates a net activation of coagulation.

177

178   *CNT treatment promotes TF production in THP-1 cells via the MAPK signaling cascade*

179   To assess the involvement of monocytes in TF production, the time course of *TF* mRNA

180   expression in THP-1 cells stimulated with CNT was investigated. CNT treatment induced no

181   increase in *TF* mRNA expression for 2 h after incubation; however, a significant increase in *TF*

182   mRNA expression was observed after 4 h ($p < 0.05$, Fig. 4a). Therefore, subsequent

183   experiments using THP-1 cells were performed after 4 h of incubation with CNT.

184   In addition, we investigated a signaling pathway that might be involved in TF generation

185   in monocytes; the p44/42 MAPK signaling pathway exerts diverse effects in many cell types [13].

186   To elucidate the involvement of MAPK signaling in CNT-mediated TF production in THP-1 cells,

187   PD98059, which specifically inhibits MEK-1-mediated activation of MAPK, was used. CNT

188   induced phosphorylation of p44/42 MAPK, whereas PD98059 inhibited CNT-induced

189   phosphorylation of MAPK (Fig. 4b). Furthermore, PD98059 suppressed *TF* mRNA expression in

190   THP-1 cells (Fig. 4c) and EV-TF release in conditioned media (Fig. 4d).

191

192   **Discussion**

193   In the present study, we show the possible involvement of monocyte-derived EV-TF in the

194   hypercoagulable state induced by CNT.

195   EV is a collective term for vesicles with a heterogeneous lipid bilayer structure secreted

196   by most living cells; EVs facilitate intercellular communication by acting as a mediator for

197   transporting various biological substances [14]. In addition, the pro-coagulant activity of EVs is

198   evident from the association of increased EV levels with increased risk of thrombosis in clinical

199   settings [5,15]. The pro-coagulant properties of EVs can be attributed to the surface expression

200   of pro-coagulant proteins, such as TF and phosphatidylserine (PS) [5]. However, EVs derived

201   from different blood cells differ in their potential to induce thrombin generation. Platelets are

202   considered to have a higher capacity for thrombin generation when compared to other blood cell

203   types [16]. The difference may be attributed to PS expression, which is expressed more

204   abundantly on platelets than on other blood cells, including monocytes [17]. However, the widely

205   accepted cell-based theory of coagulation states that the initiation phase of coagulation is

206   triggered on TF-bearing cells or TF-containing EVs [6]. Therefore, the physiological activator TF

207   is key in the process of blood clotting. A small quantity of thrombin generated in the initiation

208   phase of coagulation strongly activates platelets, leading to the expression of PS [18].

209   Based on this critical role of TF in the coagulation process, we first explored whether

210   CNT increases EV-TF activity using whole blood samples. We observed enhanced EV-TF

211   activity in the presence of CNT, suggesting that CNT might stimulate blood components to

212   express TF. Next, the ROTEM assay revealed that CNT induced a hypercoagulable state in

213   whole blood. TAT ELISA analysis further demonstrated that CNT-induced coagulation led to

214   thrombin generation. These results suggest that EV-TF might be involved in the early stage of

215   the coagulation cascade by which CNT induced a hypercoagulable state.

216   While analyzing TF expression, we focused on monocytes because they represent a

217   leading candidate as a source of EV-TF in whole-blood components [19,20]. Gene expression

218   analysis revealed an increase in *TF* mRNA only after 4 h of incubation of THP-1 cells with CNT,

219   suggesting that CNT might promote the de novo synthesis of TF. Furthermore, inhibition of both

220   *TF* mRNA expression and EV-TF production in THP-1 cells by PD98059, a specific MEK inhibitor,

221   indicated that monocyte-derived EV-TF production might be enhanced via the MAPK pathway.

222   The findings of the present study together with our previous findings indicate that CNT

223   treatment increases TF expression in vascular endothelial cells [4] and establish the

224   procoagulant properties of CNT. Virchow's triad describes three factors that contribute to

225   thrombosis: endothelial damage, stasis of blood flow, and hypercoagulability [21]. Our results

226   imply that CNT administration satisfies at least two of the three conditions of the Virchow's triad,

227   that is, hypercoagulation and endothelial cell damage, which may easily lead to thrombosis in

228   areas of static or turbulent flow with altered hemodynamics.

229   Cardiac glycosides, including CNT, are a class of organic compounds that increase the

230   output of the heart and decrease its contraction rate by inhibiting the cellular sodium-potassium

231   ATPase pump, and are hence used for the treatment of cardiac disorders, such as congestive

232   heart failure. Notably, treatment with cardiac glycosides has been reported to reduce the

233   development of carcinomas, prevent cancer recurrence, and improve survival in patients with

234   malignancies, such as breast, liver and colon cancers, [22–24]. CNT, a cardiac glycoside, has

235   recently emerged as a potential anticancer agent [25–27]. However, CNT may cause concerns

236   related to its procoagulant activity as well as cardiotoxic side effects arising from the narrow

237   therapeutic index when used as a therapeutic agent. Previous studies have shown that the CNT

238   concentrations that exert an anticancer effect are slightly lower than or equal to the

239   concentrations used in the present study [27,28]. As patients with cancer are prone to thrombotic

240   complications, it will be necessary to consider potential hypercoagulability in clinical applications

241   of CNT, even within the therapeutic index.

242   This study had several limitations. First, we evaluated monocytic human THP-1 cells,

243   rather than monocytes. This cell line has been extensively used to study the properties of

244   monocytes and can simulate potential responses. Although THP-1 cell responses should be

245   validated via *in vivo* studies [29], we believe that these results reflect the properties of the

246   monocytes themselves. Next, blood cells other than monocytes, such as different types of

247   leukocytes or platelets, were not examined as the source of TF-EVs in this study. Other blood

248   cells may represent a source of TF-EVs; however, as monocytes are considered a primary

249   source of TF-EVs among blood cells in many clinical conditions [20,30], we believe that

250   experiments with monocytes alone are sufficient. Finally, the findings obtained in this study were

251   based on *ex vivo* experiments. Further animal experiments are warranted to clarify the effects of

252   CNT *in vivo*.

253   In conclusion, the present study demonstrated that CNT shortened the coagulation time

254   of whole blood and increased EV-TF activity in monocytes through the MAPK pathway.

255   Combined with previous findings showing that endothelial cell damage mediated by CNT

256   increases TF expression, the procoagulant properties of CNT have been further established in

257   the present study. The procoagulant properties of CNT should be considered when considering

258   clinical applications of CNT.

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260

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343

344 **Figure legends**

345 Figure 1. Effect of CNT on EV-TF activity in plasma obtained from whole blood after 4 h

346 stimulation with various concentration of CNT. (* $p < 0.05$, n = 3). CNT, convallatoxin; EV-TF,

347 extracellular vesicle-associated tissue factor.

348

349 Figure 2. Effect of CNT on whole-blood coagulation. **a**. (Top) A typical rotational

350 thromboelastometry curve. The following numerical parameter was obtained: CT plus CFT,

351 which is an indicator of whole blood clotting. (Bottom) CT plus CFT was reduced in the presence

352 of CNT at a final concentration of 100 nM. **b**. The reduction of CT plus CFT in the presence of

353 CNT was statistically significant (* $p < 0.05$, n = 6). CNT, convallatoxin; CT, clotting time; CFT,

354 clot formation time.

355

356 Figure 3. TAT levels in plasma obtained from whole blood after 4 h stimulation with or without

357 CNT at a final concentration of 100 nM (* $p < 0.05$, n = 6). TAT, thrombin-antithrombin complex;

358 CNT, convallatoxin.

359

360 Figure 4. **a.** Temporal alterations in *TF* mRNA expression in THP-1 cells cultured in the presence

361 of CNT. **b.** Western blot analysis of CNT-induced p44/p42 MAPK phosphorylation with or without

362 MAP kinase kinase inhibitor PD98059 (10 µM f.c.). **c.** Inhibitory effect of PD98059 (10 µM f.c.) on

363   CNT-induced *TF* mRNA expression. **d.** Inhibitory effect of PD98059 (10 µM f.c.) on CNT-induced

364   EV-TF activity elevation. (* $p < 0.05$, n = 3). TF, tissue factor; CNT, convallatoxin; MAPK,

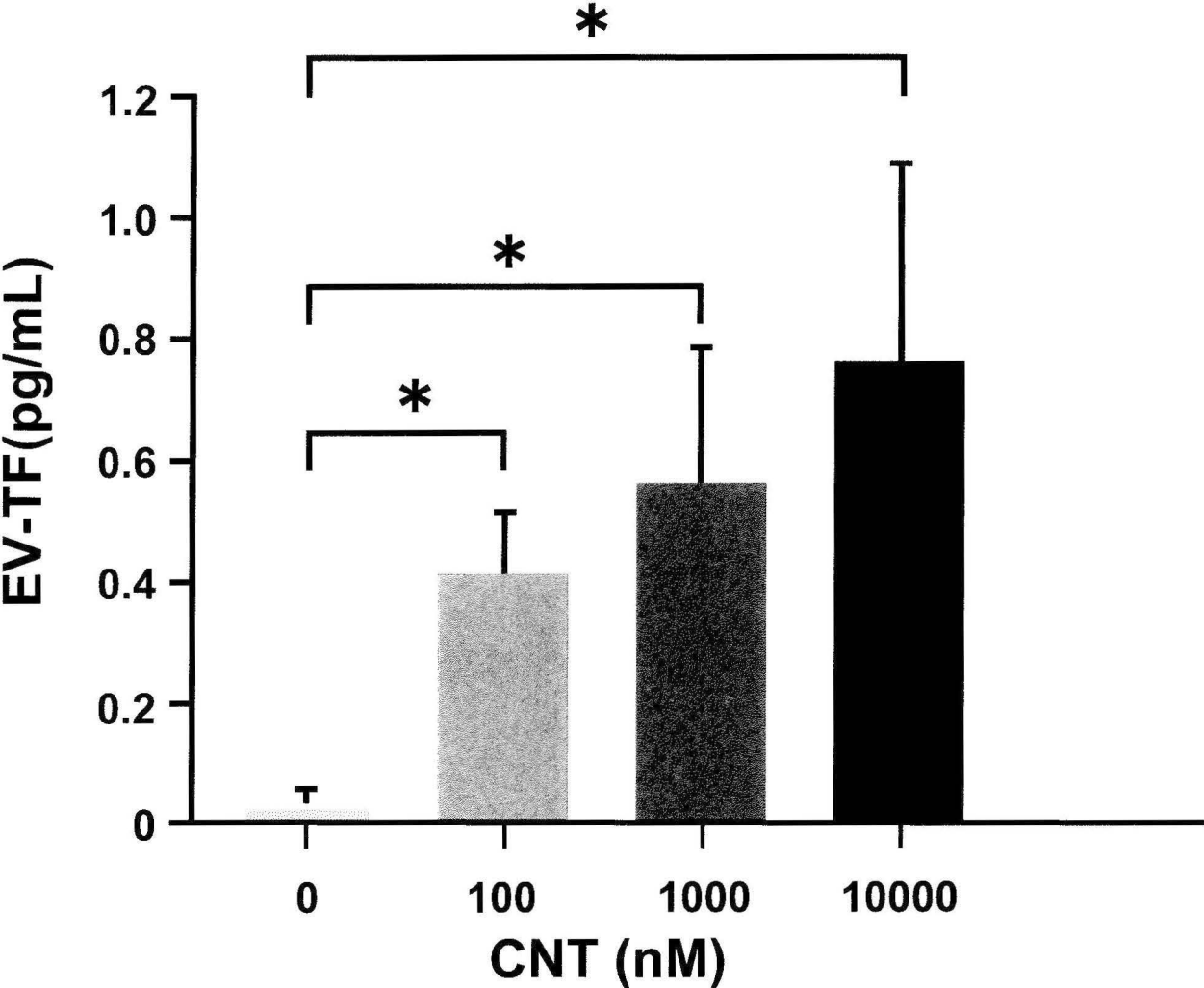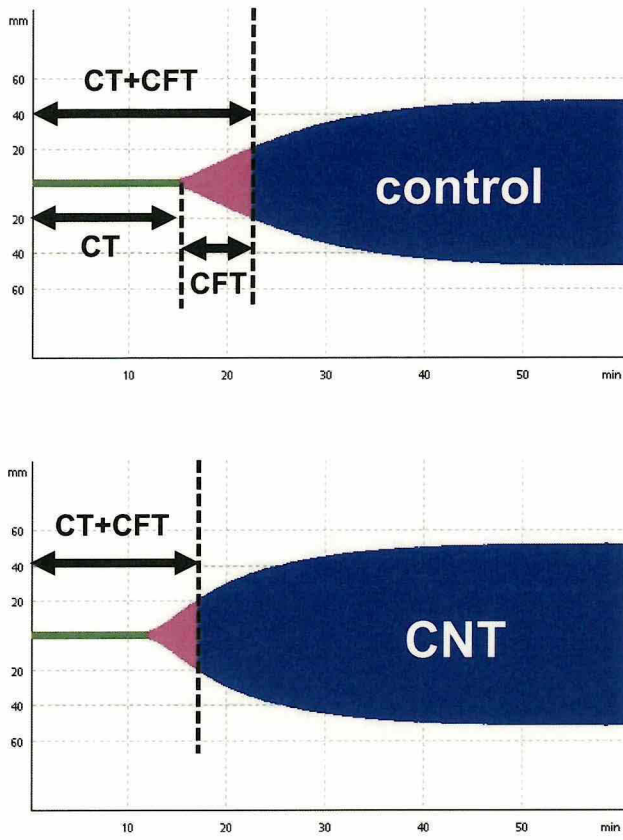365   mitogen-activated protein kinase; EV-TF, extracellular vesicle-associated tissue factor.
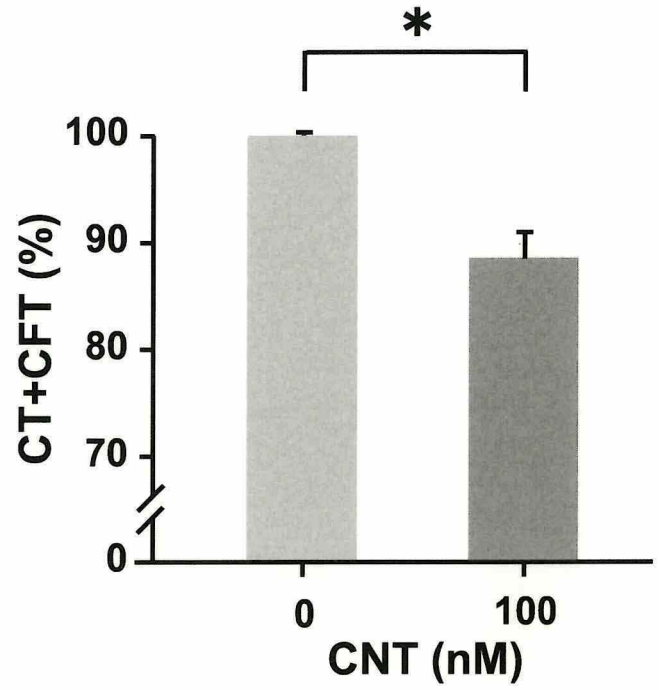
Figure 1

Figure 2

a

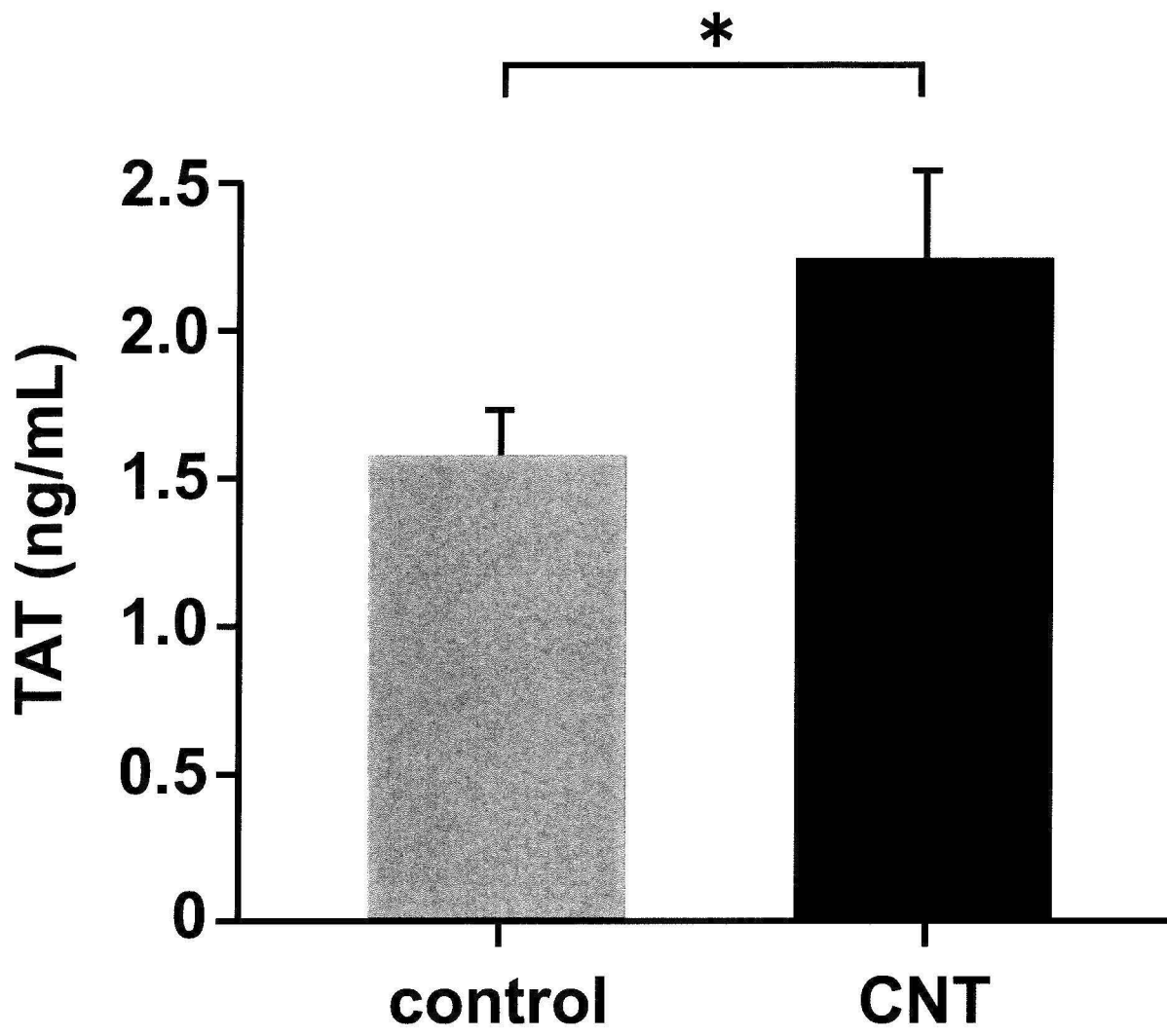## b

Figure 3

Figure 4