

Alcoholic extract of *Tarantula cubensis* induces apoptosis in MCF-7 cell line.

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Abstract

Tarantula cubensis Alcoholic Extract (TCAE) is a homeopathic agent used for treating many disorders. This study aimed to define the effects of TCAE on the breast carcinoma cell line (MCF-7). After various concentrations (10, 20, 40, 80 and 160 µl/ml) of TCAE were applied to MCF-7 cells and the human embryonic kidney cell line (HEK293), the cells were incubated for 1, 3, 6, 9, 12, 24 and 48 h, followed by analysis by MTT assays. According to the results of the MTT assays, cells treated with 20 or 40 µl/ml TCAE for 6 h were applied to apoptosis analysis by flow cytometry. Secreted levels of tumor necrosis factor alpha (TNF α), interleukin (IL)-1 β , IL-6, IL-10, Interferon- γ (IFN γ), Transforming Growth Factor- β (TGF β), and Nuclear Factor-kappa B (NF- κ B) were measured using ELISAs. TNF α and TGF β levels increased while IL-6 and IL-10 levels fluctuated in MCF-7 cells. In conclusion, our study suggests that TCAE may change the normal cancer physiology and lead to cell death by activating apoptosis in MCF-7 cells.

Keywords: *Tarantula cubensis* alcoholic extract, Theranekron, Cytokines, MCF-7, Apoptosis.

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Introduction

Tarantula cubensis Alcoholic Extract (TCAE) is a homeopathic remedy prepared from the spider *Tarantula cubensis*. The whole spider is processed and diluted with 60% alcohol for potentiation according to the rules of the "Pharmacopeia Germanica" [1]. In veterinary medicine, it is often used as an epithelialization accelerator and demarcation provider because of its oedema-relieving effects in trauma, necrotic disorders, and many infectious diseases [1-3]. TCAE also has an anti-inflammatory effect [1]. Administration of TCAE leads to regression of mammary tumors, and postoperative use of TCAE prevents the reoccurrence of tumors in dogs [4]. In addition, TCAE is effective for the treatment of oral ulcers and papillomas [5,6].

Breast cancer is frequently observed among women and is the fourth most common cause of cancer deaths [7]. One of the etiologic factors of breast cancers is chronic inflammation [8]. Cytokines play crucial roles in inflammation and are produced by tumor cells [9], which are associated with malignancy, the tumor stage, and survival [10]. They are the most significant players in tumor initiation, promotion, angiogenesis, and metastasis [10].

Nuclear factor-kappa B (NF- κ B) is a major survival factor that regulates cellular processes such as cell adhesion, immune responses, apoptosis, and proliferation. It has a regulatory role in the balance of apoptosis-proliferation in tumor cells. Moreover, the expression of inflammatory cytokines is induced by NF- κ B, and in turn, these cytokines induce activation of NF- κ B. Inhibition of NF- κ B can improve the efficacy of cancer therapies [11,12]. Studies have shown that NF- κ B plays a role in the transition from chronic inflammation to cancer [13].

Tumor Necrosis Factor- α (TNF α) is a multifunctional proinflammatory cytokine that plays important roles in the proliferation, differentiation, survival, and death of cells [14]. It is an angiogenic stimulator that promotes tumor cell migration and invasion [15,16]. Local administration of a high dose of TNF α selectively devastates blood vessels in tumors, thus showing potent anticancer effects. However, upon chronic production, TNF α acts as an endogenous tumor-promoting agent that enhances tumor growth and spread [17]. Interleukin (IL)-1 promotes tumor proliferation, invasiveness, angiogenesis, and metastasis. Because IL-1 receptor antagonist (IL-1Ra) inhibits IL-1 functions, it decreases tumor invasiveness and can be used in cancer therapy [18,19]. IL-6 is a prognostic factor in breast carcinomas and plays an important role in the development, invasion, proliferation, apoptosis, and

angiogenesis of tumors. Moreover, IL-6 is a significant contributor to tumor cell survival and drug resistance. Conversely, expression of IL-6 is not induced by breast cancer cells sensitive to drugs treatments, while its expression is produced by breast cancer cells resistant to drugs treatments [20]. IL-10 has an antitumor effect in cancer and displays a tumor regression activity [21].

Transforming Growth Factor- β (TGF β) is a major regulator in many cellular processes such as proliferation, differentiation, migration, and apoptosis. TGF β has dual functions in tumor progression [22]. TGF β as a tumor suppressor has anti-proliferative effects in the early stages of tumorigenesis, but tumor cells become resistant to this effect in later stages [22,23]. Blocking TGF β improves the intratumoral penetration of chemotherapeutic drugs and decreases angiogenic gene expression [24].

Interferon- γ (IFN γ) is a potent immunomodulatory, antiviral, and anti-proliferative cytokine. In addition, it inhibits tumor angiogenesis and shows an anticancer activity. IFN γ increases cellular susceptibility to apoptosis in tumor cells [25].

The homeostasis of cell proliferation and death is regulated by apoptosis in multicellular organisms. Therefore, treatments that affect the apoptotic threshold can change the natural progression of certain diseases such as cancer [26]. Considering these facts and the effects of TCAE on cancer, papilloma, and ulcers in clinical trials [4-6], it has been hypothesized that cancer physiology may be affected by TCAE.

The aim of the present study was to determine the effects of TCAE on cell proliferation, and apoptosis, NF- κ B expression, and secreted cytokine levels in the MCF-7 cell line, because NF- κ B, TNF α , IL-1 β , IL-6, IL-10, TGF β , and IFN γ have important roles in cancer.

Materials and Methods

Agents and cell culture

TCAE (Theranekron D6[®] Enj. Sol. Richter Pharma, Austria), breast cancer cell line (MCF-7) and the human embryonic kidney 293 (HEK293) cell line were used in this study. Cell lines were obtained from the American Type Culture Collection (ATCC).

Thiazolyl blue tetrazolium bromide (MTT) assay

To determine the cytotoxic effect of TCAE, we performed MTT assays. For the MTT assay, 5×10^3 cells in 100 μ L culture medium were added to each well of a 96-well microplate and incubated for 24 h at 37°C with 5% CO₂. The medium was removed and the cells were washed twice with PBS. To equalize the mitosis cycle, the cells were incubated in serum-free medium for 24 h (starvation). Then, the cells were treated with 100 μ L culture medium containing various concentrations (0, 10, 20, 40, 80, and 160 μ L/ml) of TCAE for 1, 3, 6, 9, 12, 24 and 48 h. At the end of each time point, 10 μ L

of a 12 mM MTT solution (#M2128; Sigma-Aldrich) was added to each well, followed by incubation for 4 h at 37°C with 5% CO₂. After the medium was removed, 50 μ L DMSO was added to each well, followed by incubation for 10 min at 37°C with 5% CO₂. The blue crystals were dissolved by gentle pipetting after incubation, and then absorbance values were measured on an ELISA reader (MWGt Lambda Scan 200, Bio-Tek Instruments, Winooski, VT, USA) at 490 nm.

Apoptosis analysis

Because TCAE-induced cytotoxicity induces different cell death pathways, such as apoptosis and necrosis, we applied Annexin V staining and flow cytometry. Annexin V distinguishes apoptotic cell death. About 1×10^6 cells were seeded into a flask with 3 ml medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. After the cells were treated with optimal TCAE concentrations (20 and 40 μ L/ml) and times (6 h) determined by the MTT assay, 5 μ L Annexin V-FITC (eBioscience ABD #BMS500FI/20) was added to each well. Cells were collected by trypsinization. The cells were incubated for 10 min at room temperature, washed with Binding Buffer (1X), and then stained with 10 ml propidium iodide (eBioscience ABD, #BMS500FI/20) (20 μ g/ml). Analysis was performed with a cell sorter (FACSAria III, BD Biosciences).

Cytokine analysis

Supernatants were obtained from MCF-7 cell treated with or without TCAE for 2, 4, 6, 8, 10 and 24 h. Secreted levels of NF- κ B, TNF α , IL-1 β , IL-6, IL-10, TGF β , and INF γ were determined by ELISAs (eBioscience, San Diego, CA, USA) as described by the manufacturer's instructions. Plates were read using the ELISA reader.

Statistical analysis

Intra-group MTT assay and apoptosis analyses were evaluated by dependent t-tests (MINITAB[®] Release 14). P<0.05 was considered as statistically significant. Cytokine levels were evaluated by analysis of variance and Duncan's post-hoc test (SPSS 19.0). P<0.05 was considered as statistically significant.

Results

MTT assay

To determine the optimal treatment time and TCAE concentration for further analyses, MTT assays of MCF-7 and HEK293 cell lines were performed using various TCAE concentrations (0, 10, 20, 40, 80, and 160 μ L/ml) for different times (1, 3, 6, 9, 12, 24, and 48 h) (Figure 1). TCAE showed an inhibitory effect on the proliferation of MCF-7 cells in concentration- and time-dependent manners.

Cell proliferation was significantly decreased by 10, 20, 40, 80 and 160 μ L/ml TCAE compared with MCF-7+TCAE and MCF-7 groups at 6 h (Figure 1). Compared with HEK293 cells under the same conditions, cell proliferation was decreased in a

dose-dependent manner following partial increases by treatment with 10, 20, 40, 80 and 160 µl/ml TCAE (Figure 1). MTT assay was repeated at least 5 times in groups.

Table 1. Effect of TCAE on TNFα, IL-1β, IL-6, IL-10, IFNγ, TGFβ and NFκB levels in MCF-7 cell line (Mean ± SE) breast carcinoma cell line, TCAE: *Tarantula cubensis* Alcoholic Extract; TNFα: Tumor Necrosis Factor Alpha; IL-1β: Interleukin-1 Beta; IL-6: Interleukin-6; IL-10: Interleukin-10; IFNγ: Interferon Gamma; TGFβ: Transforming Growth Factor Beta; NF-κB: Nuclear Factor Kappa B; Abs: Absorbance a, b: Different letters in the same row statistically are important (P<0.05).

Parameters	Group	2 h	4 h	6 h	8 h	10 h	24 h
TNFα (pg/mL)	MCF	2.35 ± 1.32 ^b	0.83 ± 0.32 ^b	0.77 ± 0.35 ^b	1.28 ± 0.69 ^b	5.01 ± 3.87 ^b	17.27 ± 8.53 ^a
	MCF+TCAE	0.81 ± 0.53 ^a	1.31 ± 0.58 ^a	0.38 ± 0.13 ^a	0.61 ± 0.32 ^a	0.61 ± 0.29 ^a	1.33 ± 0.026 ^a
IL-1β (pg/mL)	MCF	9.90 ± 0.53 ^a	9.96 ± 0.18 ^a	10.46 ± 0.55 ^a	10.03 ± 0.78 ^a	10.24 ± 0.36 ^a	10.93 ± 0.88 ^a
	MCF+TCAE	9.68 ± 0.23 ^a	10.31 ± 0.79 ^a	10.49 ± 0.37 ^a	9.68 ± 0.27 ^a	10.65 ± 0.57 ^a	12.30 ± 1.69 ^a
IL-6 (pg/mL)	MCF	7.34 ± 1.80 ^{ab}	4.25 ± 1.62 ^b	8.43 ± 2.24 ^{ab}	12.83 ± 4.37 ^a	12.96 ± 1.82 ^a	3.77 ± 1.80 ^b
	MCF+TCAE	2.63 ± 1.23 ^a	7.23 ± 2.55 ^a	10.53 ± 4.69 ^a	8.66 ± 2.44 ^a	1.73 ± 0.79 ^a	8.96 ± 3.07 ^a
IL-10 (pg/mL)	MCF	4.05 ± 0.63 ^a	3.19 ± 0.61 ^{ab}	2.50 ± 0.46 ^b	2.71 ± 0.40 ^{ab}	3.36 ± 0.30 ^{ab}	3.68 ± 0.27 ^{ab}
	MCF+TCAE	3.52 ± 0.72 ^a	5.31 ± 1.48 ^a	3.99 ± 0.89 ^a	3.52 ± 0.60 ^a	4.13 ± 0.59 ^a	4.33 ± 0.31 ^a
IFNγ (pg/mL)	MCF	0.14 ± 0.05 ^a	0.23 ± 0.10 ^a	0.40 ± 0.12 ^a	0.28 ± 0.11 ^a	0.42 ± 0.24 ^a	0.45 ± 0.08 ^a
	MCF+TCAE	0.55 ± 0.32 ^a	0.37 ± 0.08 ^a	0.56 ± 0.15 ^a	0.92 ± 0.20 ^a	0.56 ± 0.10 ^a	0.77 ± 0.25 ^a
TGFβ (pg/mL)	MCF	2480 ± 125 ^b	2324 ± 92.3 ^b	2399 ± 82.0 ^b	2450 ± 139 ^b	2438 ± 75.3 ^b	2878 ± 154 ^a
	MCF+TCAE	2369 ± 120 ^a	2399 ± 82.0 ^a	2254 ± 53.0 ^a	2426 ± 62.1 ^a	2495 ± 118 ^a	2411 ± 75.4 ^a
NF-κB (Abs)	MCF	0.39 ± 0.01 ^a	0.47 ± 0.05 ^a	0.37 ± 0.01 ^a	0.41 ± 0.03 ^a	0.46 ± 0.03 ^a	0.43 ± 0.04 ^a
	MCF+TCAE	0.41 ± 0.03 ^a	0.39 ± 0.03 ^a	0.39 ± 0.03 ^a	0.45 ± 0.03 ^a	0.39 ± 0.04 ^a	0.41 ± 0.03 ^a

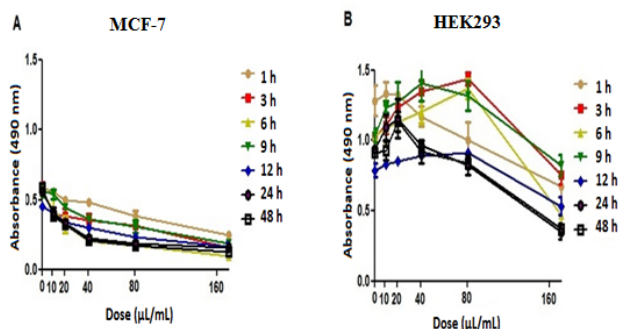


Figure 1. Result of MTT assay of TCAE on MCF-7 (A) and HEK293 (B) cell lines. Cell proliferation was significantly decreased by 10, 20, 40, 80 and 160 µl/ml TCAE compared with MCF-7+TCAE and MCF-7 groups at 6 h. Compared with HEK293 cells under the same conditions, cell proliferation was decreased in a dose-dependent manner following partial increases by treatment with 10, 20, 40, 80 and 160 µl/ml TCAE.

Apoptosis analysis

According to the results obtained from the MTT assay, treatment with 20 and 40 µl/ml TCAE for 6 h was applied for the apoptosis analysis. TCAE at concentrations of 20 µl/ml

(p=0.0293) and 40 µl/ml (p=0.0298) increased apoptosis in the MCF-7+TCAE group. In HEK293 cells, 20 µl/ml (p=0.2820) TCAE induced proliferation, whereas 40 µl/ml (p=0.0289) TCAE increased apoptosis (Figure 2). TCAE at 20 µl/ml induced the lowest apoptosis rate in HEK293, whereas same dose of TCAE induced distinct apoptosis in MCF-7 cells; hence TCAE at 20 µl/ml was used to evaluate secreted cytokine levels.

Cytokine analysis

Effects of 20 µl/ml TCAE on NF-κB, TNFα, IL-1β, IL-6, IL-10, TGFβ and IFNγ levels are shown in Table 1. Fluctuations in IL-6 and IL-10 levels, and increases in TNFα (24 h) and TGFβ (24 h) levels were found in the MCF-7 group (P<0.05). TCAE administration did not change the secreted cytokine levels of MCF-7 cells (P>0.05). To evaluate the general effect of TCAE on all cytokines, the Area under the Curve (AUC) was calculated. Therefore, we compared the AUC values of MCF-7 and MCF-7+TCAE groups. TCAE decreased the AUC values of NF-κB (-7.02%), TNFα (-87.33%), IL-6 (-34.40%), and TGFβ (-5.47%), and increased the AUC values of IL-1β (5.22%), IL-10 (23.85%), and IFNγ (70.38%).

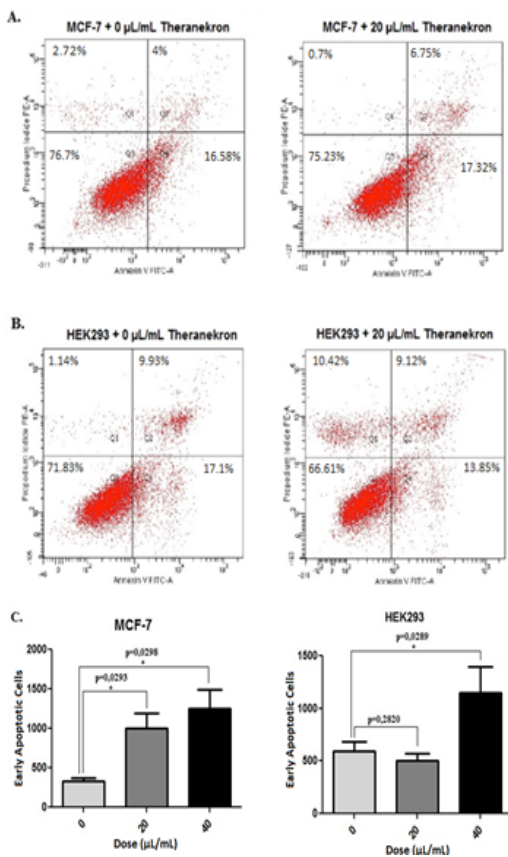


Figure 2. Apoptosis analysis in MCF-7 (A) and HEK293 (B) cells, Statistical analysis (C): TCAE at concentrations of 20 $\mu\text{L/ml}$ ($p=0.0293$) and 40 $\mu\text{L/ml}$ ($p=0.0298$) increased apoptosis in the MCF-7+TCAE group. In HEK293 cells, 20 $\mu\text{L/mL}$ ($p=0.2820$) TCAE induced proliferation.

Discussion

In veterinary medicine, TCAE is a homeopathic agent that is often used as an anti-inflammatory drug, demarcation provider, epithelialization accelerator, and oedema reliever in traumatic and necrotic disorders as well as many infectious diseases [1-3]. In addition, TCAE can also be employed in treatment of clinical sign of foot and mouth disease and endometriosis [27,28]. However, TCAE can also be applied to the therapy of some cancer types [29]. Breast cancer is one of the most frequent cancers among 40-55-year-old women. Numerous risk factors, such as age, ionizing radiation, genetic makeup, and reproductive factors, cause breast cancer [30].

In this study, the apoptotic effect of TCAE was determined in the MCF-7 cell line (Figure 2). TCAE causes apoptosis in a mammary tumor model in dogs [28]. Moreover, recent data by Dizgah et al. reported that Theranekron lead to apoptotic death through activating caspase-3 in cancer cell lines [31]. Because there is limited data associated with the apoptotic effect of TCAE, we evaluated the effect of TCAE on certain cytokines. Antiapoptotic effects can be assessed via cytokines that play important roles in the physiology of cancer. In a coordinated manner, various cytokines, such as IFN, IL-6, IL-10 and TNF, play important roles in breast carcinogenesis [21].

The NF- κB level did not statistically differ between MCF-7 and MCF-7+TCAE groups ($P>0.05$, Table 1). However, the AUC value of NF- κB was decreased by TCAE in the MCF-7+TCAE group compared with the MCF-7 group (-7.02%). NF- κB is a transcription factor that mediates the synthesis of many molecules including inflammatory agents [13]. It plays dual roles as a tumor promoter or tumor growth inhibitor [12]. However, inhibition of NF- κB has a significant therapeutic effect against breast tumor progression [32]. The antiapoptotic activity is mediated by activation of TNF, while the activity of NF- κB is inhibited by IL-10 [9,13]. Because NF- κB is an intracellular substance and shows intracellular activity in the little amounts, it does not seem to exceed the levels which make significant differences in the culture supernatant. Therefore, its level may not change by treatment with TCAE.

In the present study, the TNF α level was highest at 24 h in the MCF-7 group ($P<0.05$), but did not change significantly in the MCF-7+TCAE group ($P>0.05$) (Table 1). In addition, the AUC value of TNF α was decreased in the MCF-7+TCAE group compared with the MCF-7 group (-87.33%). TNF α is highly expressed in breast cancer [15], and has an important role in the pathogenesis of cancer [33]. It has been reported that TNF α secreted by cancer cells extends the tumor cell life through induction of NF- κB -dependent antiapoptotic molecules and acts as tumor stimulants [9]. Because the serum TNF α level correlates with the tumor stage, this cytokine reflects the severity of staging for invasive breast cancer. Serum TNF α levels at stage I and II were higher than those in controls but not statistically significant. At stages III and IV, serum TNF α levels were significantly higher than those in the control group [34]. Anti-TNF α treatment is used in several types of cancer [11]. In addition, TNF α promotes chemotherapeutic resistance in cancer [35]. The augmentation of the TNF α level at 24 h may be caused by the normal cancer physiology in MCF-7 cells for migration and invasion.

The IL-1 β level did not change significantly in MCF-7 and MCF-7+TCAE groups ($P>0.05$, Table 1). In addition, the AUC value of IL-1 β increased in the MCF-7+TCAE group compared with the MCF-7 group (5.22%). In various types of cancer, invasiveness and metastasis of tumors have been associated with IL-1 β levels [36,37]. A high level of IL-1 β correlates with a high tumor grade in invasive breast carcinoma [38]. In breast cancer, a high level of IL-1Ra and low level of IL-1 in the tumor site correlate with a good prognosis [39]. In the present study, IL-6 and IL-10 levels showed significant fluctuations in the MCF-7 group ($P<0.05$), but did not change significantly in the MCF-7+TCAE group ($P>0.05$) (Table 1). In addition, the AUC value of IL-6 decreased in the MCF-7+TCAE group compared with the MCF-7 group (-34.40%). The AUC value of IL-10 increased in the MCF-7+TCAE group compared with the MCF-7 group (23.85%). The serum IL-6 level is high in breast cancer patients. IL-6 influences proliferation, angiogenesis and apoptosis in cancer [8,40]. In breast cancer patients, a low IL-6 level indicates a good response to therapy, whereas an increased IL-6 level indicates poor responses to chemotherapeutic therapy [40]. IL-10 suppresses the production of

IL-1 β , TNF α , and IL-6 [21]. In addition, increased IL-10 might prevent tumor growth by suppressing IL-6 production. An inverse correlation exists between IL-6 and IL-10 levels in cancer patients [10]. Changes in IL-6 and IL-10 levels may be the result of normal cancer physiology in MCF-7 cells.

In this study, the TGF β level was the highest at 24 h in the MCF-7 group ($P < 0.05$), but it did not change significantly in the MCF-7+TCAE group ($P > 0.05$) (Table 1). In addition, the AUC value of TGF β decreased in the MCF-7+TCAE group compared with the MCF-7 group (-5.47%). TGF β has immunosuppressive effects [13] and can play a role in metastasis [41]. It also increases tumor vascularity [42]. TGF β suppresses the proliferation of cancer cells at early stages. However, TGF β enhances proliferation of cancer cells, tumor progression, and invasiveness at later stages [22,23]. TGF β participates in resistance to DNA-damaging chemotherapeutic agents in breast cancer cells [43]. Considering the requirement for immune system suppression in the development of cancer, it is assumed that TGF β has been secreted by the tumor cells. An increased TGF β level could arise from the effect of increasing levels of TNF α at the same time.

The IFN γ level did not change significantly in MCF-7 and MCF-7+TCAE groups ($P > 0.05$, Table 1). The AUC value of IFN γ increased in the MCF-7+TCAE group compared with the MCF-7 group (70.38%). Breast tumor cells sensitizes to apoptosis by IFN γ [44]. Gooch et al. showed significant inhibition of MCF-7 cell growth by IFN γ [45]. The IFN γ level did not change significantly in both groups, but the AUC value of IFN γ was increased by TCAE.

In conclusion, the anticancer effect of TCAE may be exerted by disturbing cancer physiology or induction of apoptosis. TCAE can be used in the treatment of cancer. However, molecular studies should be performed to determine the anticancer mechanism of TCAE.

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