Saponins from *Tribulus terrestris* L. Are Less Toxic for Normal Human Fibroblasts than for Many Cancer Lines: Influence on Apoptosis and Proliferation

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The objective of the study was to explore the influence of saponins derived from *Tribulus terrestris* L. (TT) on normal human skin fibroblasts and to compare it with their anticancer properties. In this study, [^{3}H]thymidine incorporation and MTT to assess cell proliferation and viability, respectively, and immunoblotting and HPLC analysis to explore intracellular signal transduction pathways have been used. We found that TT caused a dose-dependent decrease in [^{3}H]thymidine incorporation into the DNA of treated fibroblast compared to the untreated controls. Viability of treated cells remained within the control levels with treatment of up to 5 μg TT/ml medium. It was significantly depressed with incubation in ≥6 μg TT/ml medium with an IC50 of 12.6 μg TT/ml of cultivating media. ERK1/2 was significantly dephosphorylated at 5 mins of incubation with TT until the 48th hour, when phosphorylation slightly recovered, but was still below the control levels. In contrast, p38 and JNK phosphorylation was positively influenced, with peaks at 1 hr and 24 hrs of incubation respectively. Phosphorylation/dephosphorylation events of SAPK/MAPK clearly correlated with Mkp-1 induction. Procaspase 3 was activated after 5 mins of incubation and coincided with a rapid actin cleavage. There was a significant decrease of putrescine concentration and a concomitant increase of spermidine and spermine at 2 mins of treatment. According to our results, TT is less toxic for normal human skin fibroblasts in comparison to many cancer lines investigated in previous studies. The molecular mechanism of this cytotoxicity involves up- and downregulation of polyamines' homeostasis, suppression of proliferation, and induction of apoptosis. Further research in this field using animal models would help to explore and interpret the potential properties of TT as an anticancer supplement. Exp Biol Med 232:126–133, 2007

Key words: *Tribulus terrestris* L.; fibroblast; cytotoxicity; saponins

Introduction

Interest in herbal medicine, and in herbal anticancer therapy in particular, has progressively increased in contemporary society. One of the goals of anticancer therapy and prevention is the discovery of compounds that are relatively selective to tumor cells and therefore have reduced effects on normal cell growth.

Saponin-containing herbs possess a broad range of bioactivities and have been commonly used in folk medicine for their health-promoting properties. *Tribulus terrestris* L. is one such saponin-containing herb used from high antiquity to energize, vitalize, and improve sexual function and physical performance in men. There is a growing body of evidence for a cytostatic effect of saponins derived from different herbs against malignant cells (1–4) and anticancer properties of *T. terrestris* L. saponins (referred hereafter as TT), aside from their aphrodisiac effect (5–8). However, there still is a strong need for investigating TT effect(s) on normal human cells in order to interpret the possibility of using TT in the struggle against cancer.

Many active compounds from *T. terrestris* L. extract have been identified (9–11). Some researchers have preferred an approach of using such purified individual compounds (5, 12, 13) or their derivatives (chemically altered or naturally occurring) (1, 2) for their *in vivo* and *in vitro* studies. However, changes in the nature and biological effects of the native compound may take place during the isolation and purification process. In addition, the saponins might produce the desired effects in conjuction with other moieties. Therefore, another approach worth considering is the use of a defined saponin mixture.

The main objectives of the current research were to reveal the bioactivity of saponin mixture from *T. terrestris* L. under physiological conditions *in vitro,* to compare it

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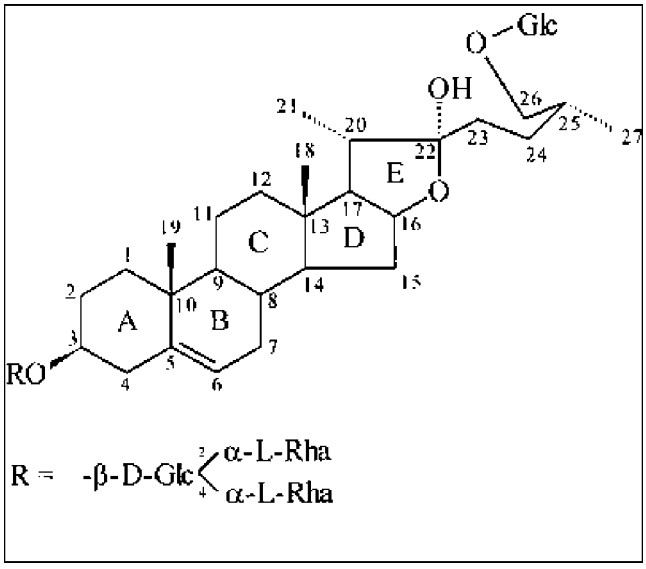

Figure 1. Chemical structure of protodioscin. Glc, glucose; Rha, rhamnose.

with saponins' anticancer properties, and to unravel, at least in part, the molecular mechanisms of the effect(s) on primary cultures from normal human skin fibroblasts.

The results show that TT is less toxic for human skin fibroblasts in comparison to a great variety of tested cancer cellular lines. The molecular mechanisms of this bioactivity involve changes in polyamine homeostasis in conjunction with antiproliferative and apoptotic molecular machinery.

Materials and Methods

Plant Materials and Purification of Saponins. *T. terrestris* L. (origin: Bulgaria) was authenticated by Dr. Kozuharova from the Department of Pharmacognosy and Botany, Faculty of Pharmacy of the Medical University of Sofia, and a voucher specimen (No. SO/104021) was deposited at the Herbarium of the Institute of Botany, University of Sofia, Bulgaria.

TT were extracted as described previously (9). In brief, the powder from aerial parts of the herb was extracted three times with 70% ethanol. The extract was evaporated to dryness under vacuum at less then 50°C. The residue was dissolved in water and extracted by aqueous butanol three times. The butanol extract was evaporated to dryness under vacuum at temperature below 50°C and was then subjected to chromatography on D101 resin. The resin was subjected to ethanol gradient elution from 0% to 100%. Fractions containing saponins were evaporated to dryness under reduced pressure and subjected to chromatography on silica gel. Saponins were eluted from the column using mixture of $CHCl_3$-MeOH-H_2O (50:10:1, v/v/v).

The contents of saponins in the fraction selected for the experiments was determined to be more than 99% by photometric analysis described previously (14). In addition, the fraction was standardized on base of protodioscin contents by RP-HPLC as described previously (15), using commercially available protodioscin (ChromaDex, Inc., Santa Ana, CA) as an external standard. Chemical structure of protodioscin is shown in Figure 1.

Materials. Dulbecco's modified Eagle's medium (DMEM), 3-(4–5-dimethyl thiasol-2-yl)-2–5-diphenyl tetrazolium bromide thiazolyl blue (MTT), fetal bovine serum (FBS), bovine serum albumin (BSA), anti-p-p38 antibody, anti-ERK 1 antibody, anti-p-ERK 1/2 antibody, anti-p-JNK antibody, anti-MKP-1 antibody, anti-Actin antibody, [^{3}H]thymidine (Sigma-Aldrich, St. Louis, MO), and anti-caspase 3 (Santa Cruz Biotechnology, Santa Cruz, CA).

Fibroblast Isolation and Cell Culture. Fibroblast cultures were established from skin of two healthy human subjects, a 34-year-old male and a 54-year-old female. Explants were obtained by surgical removal under local anesthesia, after informed consent was obtained and with approval of the local ethics committee. Epidermis and subdermal fat were removed from sterile biopsies of normal skin. The specimens were minced into pieces of 1 to 2 mm^3 in sterile tissue culture dishes and gently overlaid with DMEM supplemented with 10% FBS. Explants were incubated at 37°C in a humidified CO_2 incubator for 10 to 14 days and fed every 3 days. Fibroblasts were harvested from primary cultures by trypsin treatment and replated. Cell cultures from both donors were used at passage two to four unmixed.

Cell Proliferation Assay. Fibroblasts were plated in 24-well plates at 1.2×10^4 cells/well with DMEM supplemented with 10% FBS for 48 hrs. Cells were then starved for 24 hrs in serum-free medium supplemented with 0.1% BSA and subsequently treated with different TT concentrations (0, 0.06, 0.6, 6, 20, 60 μg/ml medium) for 20 hrs using cultivating media as a solvent. At the end of the corresponding treatment, 0.250 μCi [^{3}H]thymidine/well was added and cells were subsequently grown for an additional 4 hrs. The cells were then washed in cold phosphate-buffered saline (PBS) three times, fixed in 5% trichloroacetic acid (TCA), and dried with 70% ethanol. Finally, 250 μl of 0.2 *M* NaOH was added, allowed to stand for 15 mins, and then neutralized with 250 μl HCl acid; 150 μl of each well content was then counted in 2 ml of scintillation mix.

Cell Viability Assay. Fibroblasts were plated in 24-well plates at 1.2×10^4 cells/well, with DMEM supplemented with 10% FBS and cultivated to confluence. When cultures were confluent, TT was added in different concentrations (0, 0.06, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 10, 20, and 60 μg/ml medium) using the cultivating media as a solvent. Cells were incubated with TT for 24 hrs. Viability was monitored by MTT assay. An MTT stock solution 5 mg/ml was prepared, and a quantity was added to each culture that was equal to one tenth of the original culture volume and incubated for 4 hrs. At the end of the incubation period, medium was removed and formazan complex was solubilized with dimethyl sulfoxide (DMSO). Absorbance

of the complex was measured at a wavelength of 570 nm with background subtraction at 630–690 nm.

Western Blot Analysis. Fibroblasts from normal skin were plated into 100 mm Petri dishes (Nunc) at 1.2×10^4 cells/cm^2 with DMEM supplemented with 10% FCS and cultivated to confluence. At confluence, 6 μg TT/ml of medium was added and experiments were stopped at different times (0 mins, 5 mins, 10 mins, 30 mins, 1 hr, 24 hrs, 48 hrs, 72 hrs). At the end of the respective treatment, cells were lysed with a buffer containing 1% Nonidet P-40, 0.1% sodium deoxycholate, 150 m*M* NaCl, 50 m*M* Tris-HCl, pH7.5, 1 m*M* phenylmethyl sulfonyl fluoride, 0.2 U/ml aprotinin, 10 m*M* $Na_4P_2O_7$, 10 m*M* NaF, 4 m*M* ethylenediaminetetraacetic acid (EDTA), and 2 m*M* Na_3VO_4. The protein content was measured by the Bradford assay using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA). Cell lysates (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with 5% nonfat milk and overnight incubation at 4°C in the presence of anti-p-p38, anti-ERK 1, anti-p-ERK 1/2, anti-p-JNK, anti-MKP-1, anti-caspase 3, and anti-actin antibodies, the membrane was further processed using secondary antibodies.

Prior to the MKP-1 experiment with TT, cells were cultivated in a serum-free medium for 24 hrs to eliminate the influence of growth factors. Since the proteins used frequently as a loading control, including actin, are known caspase 3 substrates (16), we used ERK 1 as a loading control in our experiments.

Polyamine Analysis. Fibroblasts were plated into 100 mm petri dishes (Nunc) at 1.2×10^4 cells/sm^2 with DMEM supplemented with 10% FCS and cultivated to confluence. After that, 6 μg TT/ml medium was added, and experiments were stopped at different time (0 mins, 2 mins, 5 mins, 10 mins, 30 mins, 1 hr, 24 hrs, 48 hrs, 72 hrs). Cells were collected at the end of the respective experiments and washed three times with 20-ml aliquots of ice-cold PBS. The resulting pellets were resuspended in 0.4 ml of chilled 0.3 *M* perchloric acid and subjected to two cycles of freeze-thawing. After centrifugation at 12,000 *g* for 5 mins, 0.3 ml of the clear supernatant was used for polyamine analysis, whereas the pellet was dissolved in 0.4 ml of 0.3 *M* NaOH for protein determination. Putrescine, spermidine, and spermine were quantified by HPLC.

Statistical Analysis. Results were expressed as means ± SEM, and are representative of at least two separate experiments performed at least in triplicate. Statistical analysis was performed using one-way ANOVA, with a level of significance of difference set at $P < 0.05$.

Results

TT Depresses [^{3}H]Thymidine Incorporation in a Dose-Dependent Manner. To determine the effect of TT on proliferation of normal skin fibroblasts, cells were treated with different TT doses for 24 hrs. This experiment showed a dose-dependent decrease in [^{3}H]thymidine incorporation into the DNA of treated fibroblasts compared to the untreated controls (Fig. 2A). During incubation with 0.06 μg TT/ml medium [^{3}H]thymidine incorporation was 73.13 ± 7.2% of controls ($P < 0.0001$) and gradually reached 13.75 ± 2.79% with treatment of 60 μg TT/ml medium ($P < 0.0001$).

TT Depresses Fibroblast Viability. The effect of TT on cellular viability was determined by incubating fibroblasts with different TT dilutions, as described in "Materials and Methods," for 24 hrs (Fig. 2B). Viability of treated cells remained within the control levels with treatment of up to 5 μg TT/ml medium. However, it was significantly depressed (66.95 ± 4.46% of controls [$P < 0.0001$]) with incubation in ≥6 μg TT/ml medium and decreased gradually thereafter.

Half maximal inhibitory concentration (IC50) of TT was determined to be 12.6 μg TT/ml of cultivating media (BioDataFit, Chang Bioscience, Inc., Castro Valley, CA).

Rapid Effect on MAPK/SAPK Protein Kinase Subfamilies. A striking decreasing effect of TT on ERK1/2 phosphorylation was observed in the fifth minute of incubation 1.82 ± 0.196% of controls ($P < 0.0001$) (Fig. 3A and B(b)). This effect remained unchanged until the 48th hour of incubation, when ERK phosphorylation slightly recovered to 48.35 ± 1.34% ($P < 0.0001$), but was still below the control levels at 72 hrs of treatment, 58.84 ± 2.16% ($P < 0.0001$). In contrast, p38 phosphorylation was increased and reached a sharp peak after 1 hr of incubation, 210.97 ± 1.53% of controls ($P < 0.0001$), and decreased gradually until 72 hrs, when it fell slightly below the control levels (Fig. 3A and B(a)). p-JNK had a fate similar to that of p-p38, but with a gradual increase of phosphorylation and a later peak at 24 hrs, 212.68 ± 1.62% ($P < 0.0001$) (Fig. 3A and B(c)). Phosphorylation/dephosphorylation events of SAPK/MAPK correlated with rapid MKP-1 induction, which was 203.68 ± 0.33% ($P < 0.0010$) at 5 mins and reached a peak of 354.28 ± 0.73% of controls ($P < 0.0001$) at 3 mins of incubation with TT (Fig. 3A and B(d)).

Early Activation of Procaspase 3 and Corresponding Actin Cleavage. Cleavage of procaspase 3 (activation) was found after only 5 mins of treatment, 58.06 ± 1.32% ($P < 0.0001$), which reached 88.66 ± 0.90% ($P < 0.0001$) at 72 hrs of incubation (Fig. 4A and B(a)). As a consequence of procaspase 3 activation, actin was also cleaved. Actin degradation was 69.57 ± 3.13% ($P < 0.0001$) at 5 mins, and following procaspase 3 activation reached 99.10 ± 1.28% ($P < 0.0001$) at 72 hrs of treatment (Fig. 4A and B(b)). These events correlated with morphological changes in the fibroblasts, beginning with a normal fusiform appearance and shrinkage with time in TT treated cells (not shown).

Changes in Polyamine Levels Correlate with Antiproliferative and Apoptotic Effect. We found a

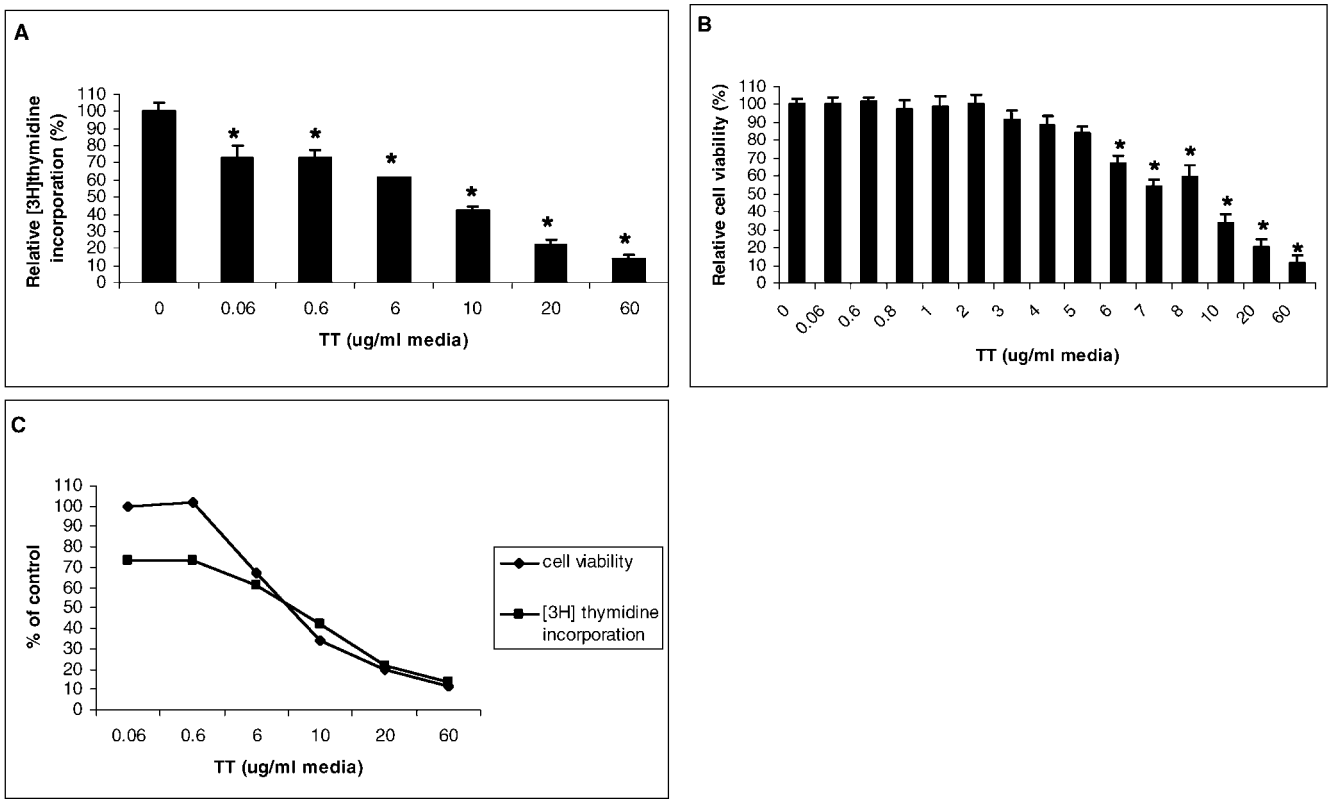

Figure 2. Influence of TT on fibroblasts' proliferation and viability. (A) [^{3}H]thymidine incorporation was significantly depressed with 0.06 μg TT/ml medium incubation 73.13 ± 7.2% of controls and gradually reached 13.75 ± 2.79% with 60 μg TT/ml medium. (B) Cellular viability decreased significantly following incubation with ≥6 μg extract/ml medium 66.95 ± 4.46% of controls. (C) Proliferation ([^{3}H]thymidine incorporation) and cell viability curves converge at a TT concentration of ~6 μg. *$P < 0.0001$ compared to controls.

significant decrease in putrescine concentration, 2.15 ± 0.38 n*M*/mg protein ($P = 0.0016$) (Fig. 5 A) and a concomitant increase in spermidine, 3.04 ± 0.13 n*M*/mg protein ($P < 0.0001$) (Fig. 5 B) and spermine 4.69 ± 0.36 ($P = 0.0003$) (Fig. 5 C) at 2 mins of treatment of fibroblasts with TT. Interestingly, 3 mins later (5 mins of treatment) polyamines returned to their initial (control) levels. At 30 mins of incubation, however, putrescine concentration increased,

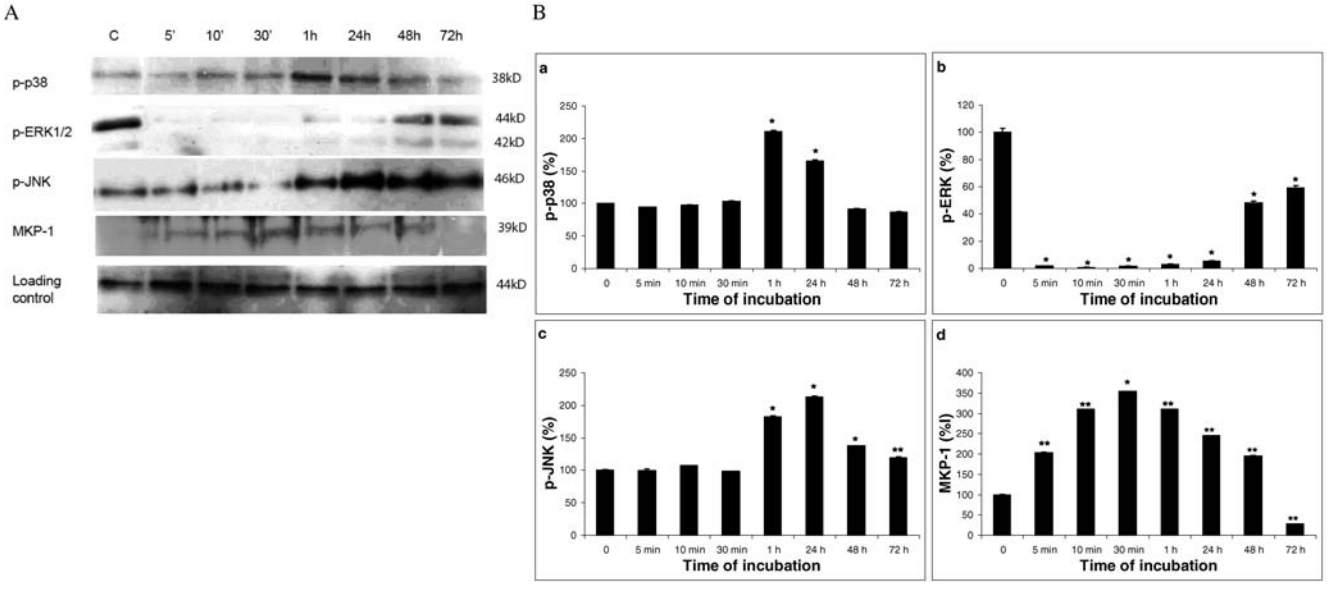

Figure 3. Effects of TT on MAPK/SAPK. (A) Immunoblot demonstrating a rapid MKP-1 induction with a powerful p-ERK1/2 dephosphorylation and an increase in p-p38 and p-JNK phosphorylation (here C is control). (B) Densitograms of a) p-p38, b) p-ERK1/2, c) p-JNK, and d) MKP-1. *$P < 0.0001$, **$P < 0.02$ compared to 0 (control) time.

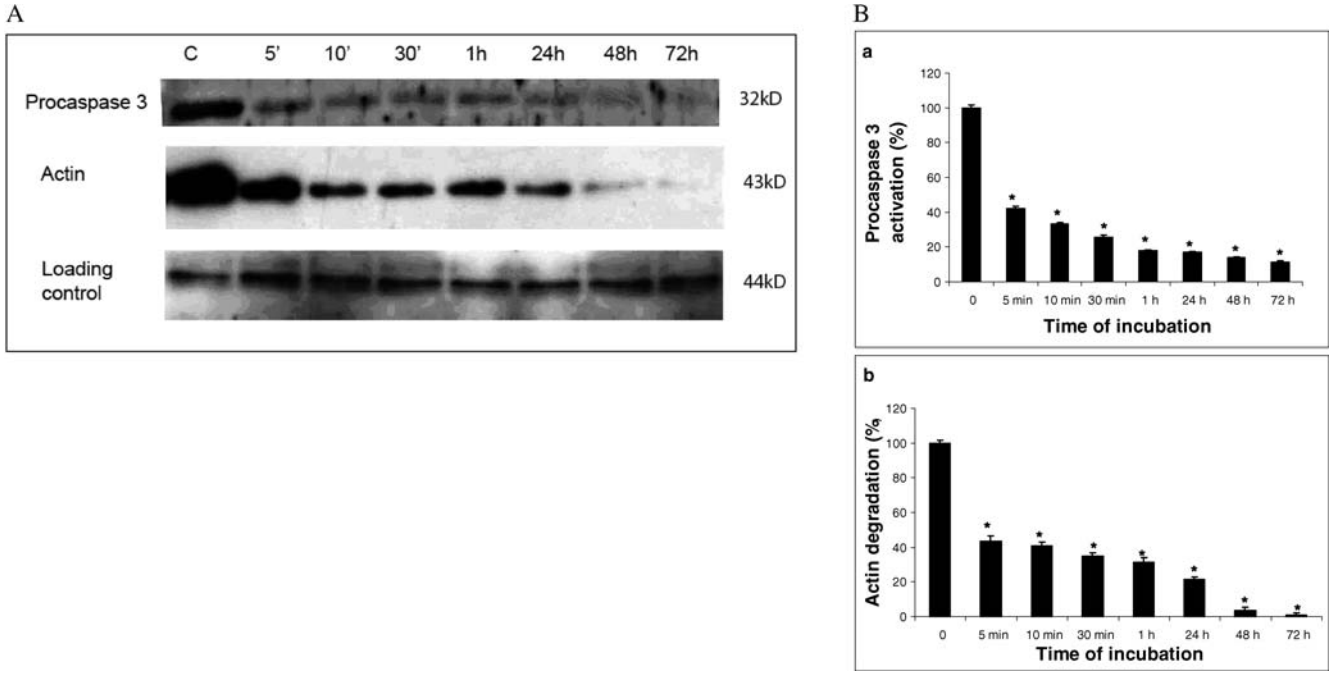

Figure 4. Influence of TT on activation of procaspase 3 and actin cleavage. (A) Immunoblot reveals a correlation between rapid procaspase 3 activation and actin degradation (here C is control). (B) Densitograms of a) procaspase 3 activation, and b) actin cleavage. *$P < 0.0001$ compared to 0 (control) time.

with a peak of 5.51 ± 0.23 n*M*/mg protein ($P = 0.0098$), whereas spermidine and spermine decreased to 0.41 ± 0.14 ($P < 0.0001$) and 1.69 ± 0.05 ($P = 0.0019$) respectively, and then all returned again to the control range. Subsequently, spermidine and spermine concentrations dropped below the controls at 48 hrs and gradually reached 0.15 ± 0.05 n*M*/mg protein ($P < 0.0001$) and 1.09 ± 0.01 n*M*/mg protein ($P = 0.0002$) at 72 hrs of the treatment respectively.

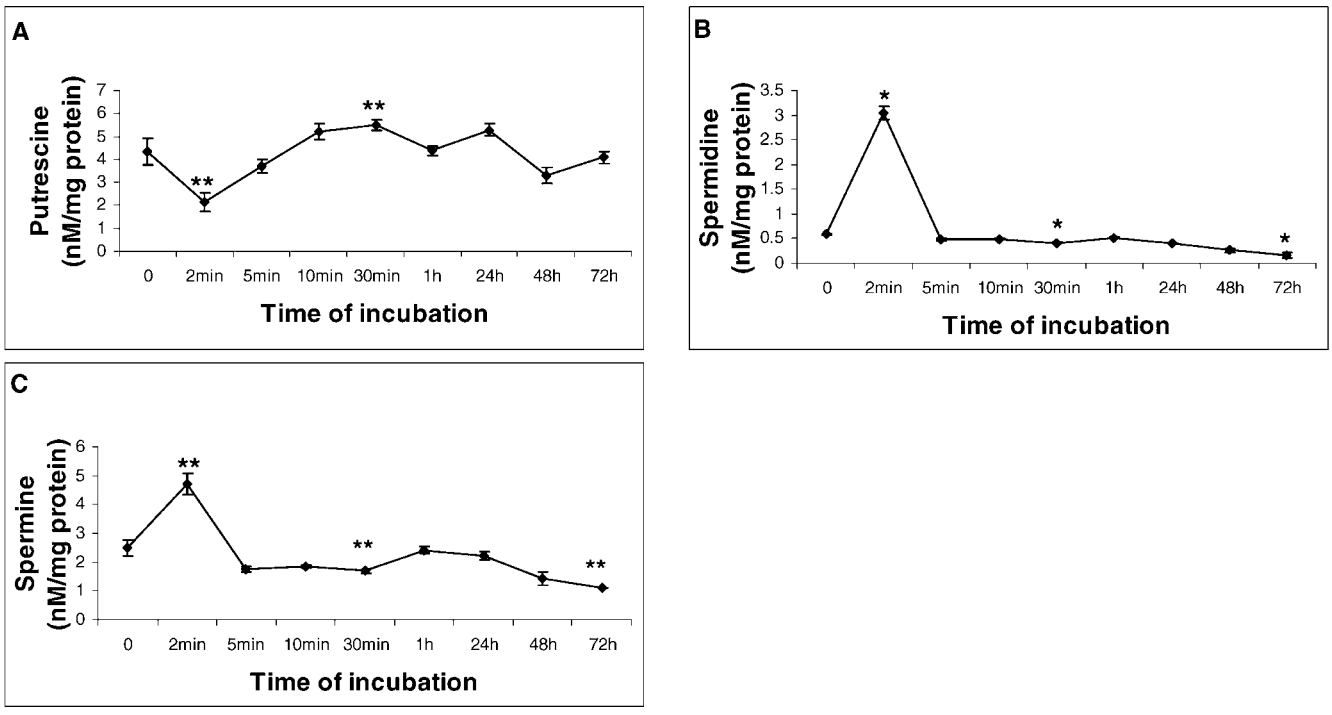

Figure 5. Effect of TT on polyamines' homeostasis. (A) Putrescine concentration decreased significantly 2.15 ± 0.38 n*M*/mg protein at 2 mins of incubation accompanied by increase in (B) spermidine 3.04 ± 0.13 n*M*/mg protein, and (C) spermine 4.69 ± 0.36. There was an inversion of changes at 30 mins with an increase of (A) putrescine and decrease of (B) spermidine, and (C) spermine followed by return to control levels. At 48 hrs of incubation spermidine and spermine dropped below the controls and reached 0.15 ± 0.05 and 1.09 ± 0.01 n*M*/mg protein at 72 hrs, respectively. *$P < 0.0001$, **$P < 0.01$ compared to 0 time.

Discussion

The goal of the current study was to explore some aspects of the effect of TT under physiological cellular conditions using primary cultures of normal human skin fibroblasts as a cellular model. The results of our research and the data from the previous studies investigating the depressive effect of TT saponins against cancer cells are considered and compared below.

First, we optimized conditions for investigating the cytostatic/cytotoxic effect in normal human fibroblasts. For this purpose, the effects of TT were determined by MTT analysis and [^{3}H]thymidine incorporation, as described in "Materials and Methods." The results obtained suggested that TT *in vitro* has cytostatic properties against normal human skin fibroblasts in concentrations ranging from 0.06 to 5–6 μg/ml of cultivating media, and increasing the concentration over 6–7 μg causes cytotoxicity with an IC50 of 12.6 μg TT/ml of cultivating media. The morphology of cells treated for 24 hrs with different cytostatic TT dilutions (0.06–5 μg) was indistinguishable from the typical fusiform morphology of the controls, whereas incubation with cytotoxic TT concentrations (≥6 μg) led to apoptotic-like shrinkage and vacuolization, and higher TT concentrations (≥20 μg) led to lysis within hours (data not shown). Despite the apoptotic-like morphological changes observed, no obvious cellular lysis was observed even at 72 hrs of incubation with 6 to 9 μg TT/ml media.

Here, we reveal the dose-dependent cytostatic/cytotoxic effect of TT against normal human cells aside from their known anticancer properties. The data from recent studies considering TT anticancer activity (5, 7, 8) has shown an IC50 ≤ 8.2 μg TT/ml of cultivating media for a great variety of human cancer cell lines, including hepatoma (BEL-7402), breast cancer (Bcap-37), melanoma malignum (SK-MEL), oral epidermoid carcinoma (KB), breast ductal carcinoma (BT-549), and ovary carcinoma (SK-OV-3). Although there was no fibrosarcoma among the investigated malignant cellular lines, the presence of a human epidermoid carcinoma line (KB) and the equivalent methods used in those studies (5) serve as an alternative for a relatively precise comparison of TT cytotoxicity in physiological and pathological conditions. According to our MTT assay results, it seems that TT is less toxic for normal human skin fibroblasts with an IC50 of 12.6 μg TT/ml of cultivating media in comparison to the vast number of investigated cancer lines (5, 7, 8).

The following observations and findings have supported additionally the estimation of working TT concentration. First, even incubation with the lowest dose of 0.06 μg TT resulted in a significant depression of [^{3}H]thymidine incorporation into cellular DNA with an unchanged viability. Second, while [^{3}H]thymidine incorporation progressively decreased, cellular viability remained within control levels with TT dilutions up to 5 μg. Further increase of TT concentration (≥6 μg), however, caused significant loss of viability. Third, [^{3}H]thymidine incorporation reached control levels 24 hrs after switching the cells cultivated with 0.06–5 μg of TT to TT-free media, but was irreversibly depressed with treatment concentrations of ≥6 μg (data not shown). Finally, as shown in Figure 2C, [^{3}H]thymidine incorporation and viability curves intersect near a TT concentration of 6 μg and proceed thereafter almost parallel.

Put together, this indicates that ~6 μg of TT represents the transition point between cytostatic and cytotoxic properties *in vitro* and the most appropriate concentration for exploring the intracellular events resulting from treatment.

The results of the MTT and [^{3}H]thymidine tests, as well as morphological observation, imply at least two possible means of TT action: (i) affecting the proliferation, and/or (ii) inducing apoptosis. Hence, the following steps for tracing the probable intracellular antiproliferative and/or apoptotic events were undertaken.

In fibroblasts, proliferation is regulated positively by the p42/44^{MAPK} (ERK1/2) and negatively by p38/JNK^{MAPK} pathway (17, 18). p38 and ERK1/2 interact physically to form a perinuclear complex, and their activities are oppositely regulated in response to different proproliferative or proapoptotic signals (19). Therefore, the possible effect of TT on ERK1/2 and the two other MAPK family members, p38 and JNK (SAPK), was investigated. As was anticipated, they were oppositely influenced. p-ERK1/2 was significantly dephosphorylated at 5 mins of incubation with TT until 48 hrs, when phosphorylation slightly recovered, but was still below the control levels. In contrast, p38 and JNK phosphorylation were positively influenced, with peaks at 1 hr and 24 hrs of incubation respectively. In order to explore this rapid p-ERK1/2 dephosphorylation event, we investigated MAPK phosphatase-1 (MKP-1) expression, because MKP-1 has been shown to dramatically inhibit fibroblast proliferation *via* p-ERK1/2 dephosphorylation and has been induced within minutes following growth factor stimulation (20). Although positive effect of growth factors was avoided by cultivating cells in a serum-free medium 24 hrs prior to the experiment, we observed a rapid expression of MKP-1 at 5 mins of incubation, with TT almost exceeding twice the control level. It reached a peak at 30 mins, decreasing gradually thereafter. There was a clear correlation between MKP-1 expression and the phosphorylation/dephosphorylation events of SAPK/MAPK caused by TT.

The physiological role of SAPK activation in cell survival and apoptosis is still controversial, being suggested to have an antiapoptotic (21), proapoptotic (22), or no effect (23) on these processes. However, the intracellular events observed in the current study together with cells' apoptotic morphological changes hinted at a possible involvement of a SAPK-dependent apoptosis initiation.

Hence, we proceeded with the assessment of the caspase 3 activation as one of the key executioners of apoptosis.

Contrary to our expectations, however, the activation of caspase 3 preceded the activation of p38 and JNK. Almost 60% of caspase 3 was activated at 5 mins and reached approximately 90% at 72 hrs of incubation with TT. The rapid apoptosis induction and caspase 3 activation, in particular, was further confirmed by the accompanying cleavage of actin. Approximately 56% of actin was cleaved at 5 mins and gradually reached 96% at 48 hrs of TT treatment and correlated clearly with caspase 3 activation (Fig. 4). Obviously, other apoptosis initiation pathways are involved and caspase 3 activation, in particular, is SAPK independent in this experiment.

On the one hand, we observed an early rapid MKP-1 induction followed by p-ERK 1/2 dephosphorylation with accompanying caspase 3 activation and rapid actin cleavage. On the other hand, there was a later effect represented by increasing phosphorylation of stress-activated protein kinases (p38 and JNK) that seems unrelated to apoptosis initiation. These findings implied that TT's signal could somehow modify crosstalk with other signal transduction pathways and/or could have a different source. One possible explanation is the close structural similarity between steroid saponins' aglycon moiety and steroid hormones, which enables them to affect other signaling molecules, such as MAPK family members, by transcription-independent mechanisms (24, 25). The other possibility is that different and some times antagonistic biological activities of TT saponins are imparted by their oligosaccharide (glycon) moiety (26). While these studies could, at least in part, reveal a possible reason for divergent TT action, neither of them could give a satisfactory explanation for the rapid apoptotic and antiproliferative TT actions observed in our research.

Some recent studies make it increasingly clear that polyamines' ubiquitous intracellular organic cations are actively implicated in the intricate mechanism of regulating cellular apoptosis and proliferation (27–29). The polyamines have been shown to regulate the activation of caspase-3 directly and/or independently modulate upstream signaling events (30).

We evaluated the possible involvement of polyamines in the molecular mechanism of TT action and their probable role in the uncoupling of signal and early caspase 3 activation. For that particular experiment, we expanded the time-dependency design with an additional end point at 2 mins of incubation with TT in order to detect any existing relation between polyamines, apoptosis initiation, and antiproliferative effect.

We found a dramatic concussion of polyamines' homeostasis with a decrease in putrescine concentration and concomitant increase in spermidine and spermine at 2 mins of treatment with TT (Fig. 5). Surprisingly, after the initial elevation, the polyamine concentrations returned to control levels. These findings left us to conclude that polyamines played a possible initiating role in TT signal transduction and supported some recent studies showing that polyamines, cell cycle events, and apoptosis are closely connected (31–33).

Here we demonstrate a probable chronology and possible correlation of intracellular events resulting from TT treatment of normal human skin fibroblasts. We propose the following order of events: a decrease of putrescine with concomitant increase of spermidine and spermine concentrations → caspase 3 activation, MKP-1 induction → p-ERK1/2 dephosphorylation and actin degradation with a normalizing of polyamine levels → p38/JNK phosphorylation and inversion of initial changes in the polyamine concentrations.

Conclusions

The following conclusions can be made from our research: (i) TT is less toxic for normal human skin fibroblasts in comparison to a great variety of tested cancer cellular lines; (ii) molecular mechanisms of TT action(s) include rapid initiation of apoptosis and depression of proliferation involving early caspase 3 activation and p-ERK1/2 dephosphorylation, respectively; (iii) within our cellular model, p38 and JNK activation during apoptosis is TT stimulus-dependent (activation of the SAPK pathways, however, is not required for procaspase 3 cleavage, but may still be required to elicit other features of the apoptotic phenotype resulting from TT treatment); and finally (iv) it is becoming more and more evident that the polyamine system is part of a tightly regulated control machinery which makes final decisions in the choice between growth, survival, differentiation, or death of a cell. Both up- and down-regulation of polyamine levels seem to play a critical role in TT signaling pathway(s). Extending this research to studies in animals would help in exploring and interpreting the potential properties of TT as an anticancer supplement.

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1. Hu K, Yao X. Protodioscin (NSC-698 796): its spectrum of cytotoxicity against sixty human cancer cell lines in an anticancer drug screen panel. Planta Med 68:297–301, 2002.
2. Hu K, Yao X. The cytotoxicity of methyl protodioscin against human cancer cell lines in vitro. Cancer Invest 21:389–393, 2003.
3. Jiang Y, Wang N, Yao X, Susumu K. Structural elucidation of the anticoagulation and anticancer constituents from Allium chinense [in Chinese]. Yao Xue Xue Bao 33:355–361, 1998.
4. Wang SL, Cai B, Cui CB, Liu HW, Wu CF, Yao XS. Diosgenin-3-O-alpha-L-rhamnopyranosyl-(1 –> 4)-beta-D-glucopyranoside obtained as a new anticancer agent from Dioscorea futschauensis induces apoptosis on human colon carcinoma HCT-15 cells via mitochondria-controlled apoptotic pathway. J Asian Nat Prod Res 6:115–125, 2004.
5. Bedir E, Khan IA, Walker LA. Biologically active steroidal glycosides from Tribulus terrestris. Pharmazie 57:491–493, 2002.
6. Deng Y, Yang L, An SL. Effect of Tribulus terrestris L decoction of different concentrations on tyrosinase activity and the proliferation of melanocytes [in Chinese]. Di Yi Jun Yi Da Xue Xue Bao 22:1017–1019, 2002.
7. Sun B, Qu W, Bai Z. The inhibitory effect of saponins from Tribulus

terrestris on Bcap-37 breast cancer cell line in vitro [in Chinese]. Zhong Yao Cai 26:104–106, 2003.

8. Sun B, Qu WJ, Zhang XL, Yang HJ, Zhuang XY, Zhang P. Investigation on inhibitory and apoptosis-inducing effects of saponins from Tribulus terrestris on hepatoma cell line BEL-7402 [in Chinese]. Zhongguo Zhong Yao Za Zhi 29:681–684, 2004.
9. Cai L, Wu Y, Zhang J, Pei F, Xu Y, Xie S, Xu D. Steroidal saponins from Tribulus terrestris. Planta Med 67:196–198, 2001.
10. De Combarieu E, Fuzzati N, Lovati M, Mercalli E. Furostanol saponins from Tribulus terrestris. Fitoterapia 74:583–591, 2003.
11. Huang JW, Tan CH, Jiang SH, Zhu DY. Terrestrinins A and B, two new steroid saponins from Tribulus terrestris. J Asian Nat Prod Res 5: 285–290, 2003.
12. Zhang JD, Cao YB, Xu Z, Sun HH, An MM, Yan L, Chen HS, Gao PH, Wang Y, Jia XM, Jiang YY. In vitro and in vivo antifungal activities of the eight steroid saponins from Tribulus terrestris L. with potent activity against fluconazole-resistant fungal. Biol Pharm Bull 28: 2211–2215, 2005.
13. Gauthaman K, Ganesan AP, Prasad RN. Sexual effects of puncturevine (Tribulus terrestris) extract (protodioscin): an evaluation using a rat model. J Altern Complement Med 9:257–265, 2003.
14. Gyulemetova R, Tomova M, Simova M, Pangarova T, Peeva S. Determination of furostanol saponins in the preparation tribestan. Pharmazie 37:296, 1982.
15. Ganzera M, Bedir E, Khan IA. Determination of steroidal saponins in Tribulus terrestris by reversed-phase high performance liquid chromatography and evaporative light scattering detection. J Pharm Sci 90: 1752–1758, 2001.
16. Villa P, Kaufmann SH, Earnshaw WC. Caspases and caspase inhibitors. Trends Biochem Sci 22:388–393, 1997.
17. Lavoie JN, L'Allemain G, Brunet A, Muller R, Pouyssegur J. Cyclin D1 expression is regulated positively by the $p42/44^{MAPK}$ and negatively by the $p38/HOG^{MAPK}$ pathway. J Biol Chem 271:20608–20616, 1996.
18. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270: 1326–1331, 1995.
19. Efimova T, Broome AM, Eckert RL. Protein kinase Cdelta regulates keratinocyte death and survival by regulating activity and subcellular localization of a p38delta-extracellular signal-regulated kinase 1/2 complex. Mol Cell Biol 24:8167–8183, 2004.
20. Sun H, Tonks NK, Bar-Sagi D. Inhibition of Ras-induced DNA synthesis by expression of the phosphatase MKP-1. Science 266:285–288, 1994.
21. Kuan CY, Yang DD, Samanta Roy DR, Davis RJ, Rakic P, Flavell RA. The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. Neuron 22:667–676, 1999.
22. Aoki H, Kang PM, Hampe J, Yoshimura K, Noma T, Matsuzaki M, Izumo S. Direct activation of mitochondrial apoptosis machinery by c-Jun N-terminal kinase in adult cardiac myocytes. J Biol Chem 277: 10244–10250, 2002.
23. Lin A, Dibling B. The true face of JNK activation in apoptosis. Aging Cell 1:112–116, 2002.
24. Migliaccio A, Di Domenico M, Castoria G, de Falco A, Bontempo P, Nola E, Auricchio F. EMBO J 15:1292–1300, 1996.
25. Castoria G, Barone MV, Di Domenico M, Bilancio A, Ametrano D, Migliaccio A, Auricchio F. EMBO J 18:2500–2510, 1999.
26. Woldemichael GM, Wink M. Identification and biological activities of triterpenoid saponins from *Chenopodium quinoa*. J Agric Food Chem 49:2327–2332, 2001.
27. Stefanelli C, Bonavita F, Stanic I, Pignatti C, Flamigni F, Guarnieri C, Caldarera CM. Spermine triggers the activation of caspase-3 in a cell-free model of apoptosis. FEBS Lett 451:95–98, 1999.
28. Ray RM, Viar MJ, Yuan Q, Johnson LR. Polyamine depletion delays apoptosis of rat intestinal epithelial cells. Am J Physiol Cell Physiol; 278:C480–489, 2000.
29. Poulin R, Pelletier G, Pegg AE. Induction of apoptosis by excessive polyamine accumulation in ornithine decarboxylase overproducing L1210 cells. Biochem J 311:723–727, 1995.
30. Bhattacharya S, Ray RM, Viar MJ, Johnson LR. Polyamines are required for activation of c-Jun NH2-terminal kinase and apoptosis in response to TNF-alpha in IEC-6 cells. Am J Physiol Gastrointest Liver Physiol 285:G980–991, 2003.
31. Monti MG, Ghiaroni S, Pernecco L, Barbieri D, Marverti G, Franceschi C. Polyamine depletion protects HL-60 cells from 2-deoxy-d-ribose-induced apoptosis. Life Sci 62:799–806, 1998.
32. Monti MG, Ghiaroni S, Barbieri D, Franceschi C, Marverti G, Moruzzi MS. 2-deoxy-d-ribose-induced apoptosis in HL-60 cells is associated with the cell cycle progression by spermidine. Biochem Biophys Res Commun 257:460–465, 1999.
33. Kramer DL, Vujcic S, Diegelman P, White C, Black JD, Porter CW. Polyamine analogue-mediated cell cycle responses in human melanoma cells involves the p53, p21, Rb regulatory pathway. Biochem Soc Trans 26:609–614, 1998.