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AN *IN VITRO* STUDY OF CYTOTOXIC EFFECTS OF GOSSYPOL ON HUMAN EPIDERMOID LARYNX CARCINOMA CELL LINE (HEp-2)

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***Aim*: The study was aimed on an evaluation of the effect of gossypol at concentration of 25–150 μM on the level of apoptosis in the human epidermoid larynx carcinoma cell line, HEp-2, *in vitro*. *Methods*: To determine the apoptotic effects of gossypol on growth of human epidermoid larynx carcinoma cell lines (HEp-2 cells), morphological evaluation of the cells and DNA fragmentation analysis were used. *Results*: 6 h incubation of HEp-2 cells with gossypol at concentrations of 50 μM and 75 μM resulted in the increase of apoptosis rate by 26 and 23%, respectively. *Conclusion*: This preliminary study may constitute a base for *in vivo* studies of anticancer properties of gossypol.**

***Key Words:* HEp-2 cell line, gossypol, apoptosis, DNA fragmentation.**

Gossypol, a polyphenolic compound occurring in cottonseed, has antiproliferative effect on a number of cancer cells *in vitro* [1-3]. Clinical trials indicate that gossypol is showing potential as a treatment for adrenal, prostate and mammary carcinomas, gliomas, endometriosis and uterine myoma [1]. Recent evidence suggests that gossypol may affect cell cycle distribution and cell cycle regulators such as cyclin D1 and Rb in human mammary tumor cells [4]. However, we have insufficient information on the mechanism of gossypol-induced antitumor activity.

Apoptosis is characterized by internucleosomal DNA fragmentation and is associated with morphological changes including chromatin condensation, cell shrinkage, membrane blebbing and disintegration of the cell into membrane-bound fragments named apoptotic bodies. These changes in cells undergoing apoptosis are easily recognizable using electron or light microscopy. Internucleosomal DNA fragmentation may be detected by gel electrophoresis [5].

The study was aimed on the evaluation of the effect of gossypol at concentration of 25–150 μM on the level of apoptosis in the human epidermoid larynx carcinoma cell line (HEp-2) *in vitro*.

Cell line and gossypol treatment. The HEp-2 cells were supplied by the Cell Culture Collection Department, Foot and Mouth Disease Institute, Ankara, Turkey. HEp-2 cells were cultured at 37 °C in a humidified athmosphere with 5% CO_2 in RPMI 1640 medium (Biological Industries) supplemented with 10% heat-inactivated fetal calf serum, 200 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were seeded on six-well plates (Costar, USA) at a density of 5 x 10^5 cells/ml. Gossypol acetic acid is referred to in this study as gossypol (1.0 μg/ml = 1.73 μM). Since the gossypol was dissolved in ethanol, 99.5% ethanol was used as solvent control. To determine the cytotoxic effects on the morphology and growth of HEp-2 cells, gossypol was added to the cell culture at concentrations of 25–150 μM. Cell viability was assessed in 6 h after the addition of gossypol by scoring at least 100 cells for Trypan Blue exclusion [6]. Morphological evaluations of different stages of apoptosis were conducted by fixing the cells in 70% ethanol followed by staining in 10% Giemsa solution (Merck). The percentages of apoptotic cells were determined by counting at least 100 cell fields.

Detection of DNA fragmentation. HEp-2 cells were incubated in 3 ml of medium with different concentrations of gossypol for 6 h using six-well culture plates, six wells for each concentration. Cells were cedimented by centrifugation for 10 min at 1600 rpm, and the cell pellets were incubated for 1 h at room temperature in phosphate-citric acid buffer. After centrifugation at 1600 rpm for 5 min, NP-40 and RNase A were added to the supernatant for 30 min at 37 °C, then proteinase K solution was added and the mixture was incubated at 37 °C for a further 30 min. DNA samples were analysed by electrophoresis in 1% agarose gel.

Statistics. Since all samples were normally distributed according to the one-way ANOVA method, Student's *t*-test was conducted to evaluate the significance of changes in apoptosis and viability rates.

Apoptotic features were observed in HEp-2 cells after 6 h exposure to different concentrations of gossypol (Fig. 1): the rates of apoptosis and cell viability were 12 ± 1.4% and 80 ±2.9%; 26 ± 1.5% and 84 ± 2.4%; 23 ± 1.3% and 78 ± 2.4%; 10 ± 1.4% and 50 ± 5.4%; 8 ± 1.8% and 25 ± 3.7% for 25, 50, 75, 100 and 150 μM gossypol, respectively. The apoptosis and viability rates differ significantly ($p < 0.01$) compared to the control cells. Morphological examination indicated apoptosis in the treated cells, too (Fig. 2).

The study of internucleosomal DNA fragmentation in HEp-2 cells has shown that DNA fragmentation was not observed in the untreated control group (Fig. 3), whilst in the cells incubated with 50 μM and 75 μM gos-

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Abbreviations used: HEp-2 — human epidermoid larynx carcinoma cell line.

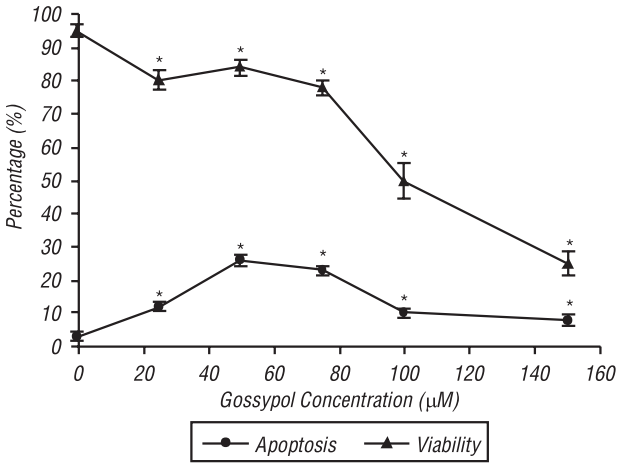


Fig. 1. Apoptosis and viability rates of HEp-2 cells after 6 h exposure to gossypol. Results are means ± S.D. for six-well cell cultures; * significant ($p < 0.01$).

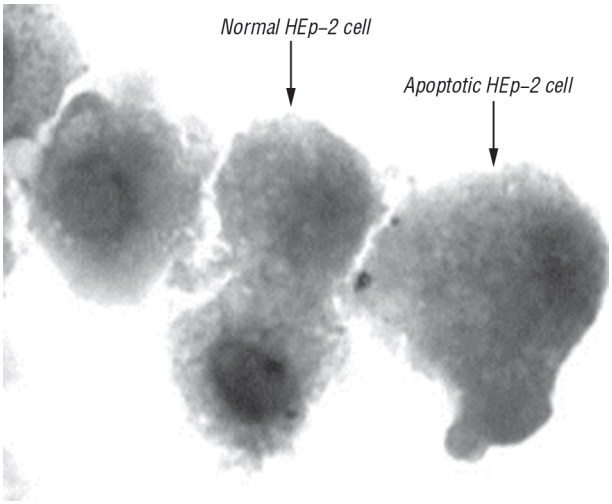


Fig. 2. HEp-2 cells exposed to 75 μM gossypol for 6 h

sipol, DNA ladder pattern confirmed high apoptosis rates, whereas in 100 μM and 150 μM concentrations the DNA ladder pattern were not as distinct (see Fig. 3). This result is consistent with the low rates of apoptosis at the higher concentrations of gossypol.

Gossypol is known as a potent inhibitor of phospholipid-sensitive Ca^{2+}-dependent protein kinase (PKC) [7] and is capable to inhibit the proliferation of a wide range of cancer cells *in vivo* and *in vitro*. Recent observations have reported its antiproliferative activity in prostate cancer cells [8]. Treatment of HT-29 human colon carcinoma cells with gossypol not only induced cell cycle arrest in the G0/G1 phase but also induced apoptosis [6]. Furthermore, treatment with 15–100 μM gossypol resulted in increased percentage of apoptotic cells in human chronic myelogenous leukemic K-562 cells [3]. In combination with alkylating agents, gossypol increased the sensitivity of drug resistant breast cancer cells [9]. In addition, gossypol increased Ca^{++} release in human hepatoma cells [10], which would point to the induction of apoptosis. Apoptosis-like DNA fragmentation was reported in HL-60 cells after 6 h incubation with 50-100 μM gossypol [7]. Recently, it has been reported that upon 6 h treatment with 50 μM gossypol, the induction of apoptosis in HL-60 cells was observed [11].

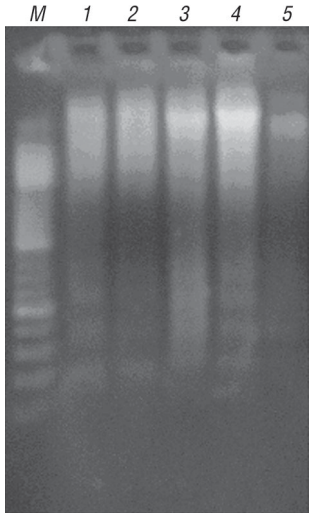


Fig. 3. 1% agarose gel electrophoresis of DNA from HEp-2 cells treated with 50 μM (lane *1*), 75 μM (lane *2*), 100 μM (lane *3*) and 150 μM (lane *4*) gossypol; M: 100 bp DNA size marker; lane *5*: control sample

In this study we found that 6 h incubation of HEp-2 cells with 50 and 75 μM concentrations of gossypol increased the rate of apoptosis by 23–26%. This preliminary study may constitute a base for *in vivo* studies of gossypol for potential anticancer therapy.

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ЦИТОТОКСИЧЕСКОЕ ДЕЙСТВИЕ ГОССИПОЛА НА КЛЕТКИ ПЛОСКОКЛЕТОЧНОГО РАКА ГОРТАНИ ЧЕЛОВЕКА ЛИНИИ НЕр-2 *IN VITRO*

***Цель*: исследовать действие госсипола в концентрации 25–150 μM на апоптоз клеток плоскоклеточного рака гортани человека линии НЕр-2 *in vitro*. *Методы*: для оценки апоптоза применяли морфологические методы и анализ фрагментации ДНК. *Результаты*: инкубация клеток в течение 6 ч с 50 μM и 75 μM госсипола приводила к увеличению доли апоптотических клеток на 26 и 23% соответственно. *Выводы*: результаты исследования могут послужить основой для исследований возможного противоопухолевого эффекта госсипола *in vivo*.**

***Ключевые слова:* клетки линии НЕр-2, госсипол, апоптоз, фрагментация ДНК.**

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