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ARAŞTIRMA / RESEARCH

Impact of gossypin on gene expression of HSP60 and HSP70 in different cancer cell lines

Gossypin'in farklı kanser hücre dizilerinde HSP60 ve HSP70'in gen ekspresyonu üzerindeki etkisi

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Abstract

Purpose: The aim of this study is to evaluate the impact of gossypin on the expression level of heat shock proteins (HSPs) genes in different cancer cells.
Materials and Methods: Cells were grown under standard culture conditions. Cancer cells were treated with different concentrations (5-100 µg/ml) of gossypin and cisplatin (50 µM) as positive control. Cell viability and effective dose range (5-100 µg/ml) of gossypin were determined by MTT at 24, 48 and 72 hours. After RNA isolation and cDNA synthesis, HSP60 and HSP70 gene expression levels were analyzed using RT-PCR. For gene expression analysis, the $2^{-\Delta\Delta ct}$ method was used.
Results: According to the MTT results, 25-50-100 µg/ml of gossypin doses were found effective on HSP60 and HSP70 gene expression levels in the cancer cell lines. Gossypin affected with dose-dependently the expression of HSP60 and HSP70 in the three cell lines. In the three cell lines, 50 µg/ml and 100 µg/ml of gossypin doses significantly reduced the expression of HSP60 and HSP70 compared to control group.
Conclusion: Our results strongly supported the anticarcinogenic effect of gossypin at various doses in different cell lines. However, we believe that further *in vivo* research and human studies are needed. Our findings suggest that gossypin could be suitable candidate agent for further investigation to develop new strategies for the prevention and/or treatment of different cancer types.

Keywords: Gossypin, HSP60, HSP70, MCF7, PC3, Hep3b

Öz

Amaç: Bu çalışmanın amacı, gossypin'in farklı kanser hücre hatlarında ısı şok proteinleri (HSP) genlerinin ekspresyon seviyesi üzerindeki etkisini incelemektir.
Gereç ve Yöntem: Hücreler, standart kültür koşulları altında büyütüldü. Kanser hücreleri, farklı konsantrasyonlarda (5-100 µg/ml) gossypin ve pozitif kontrol olarak sisplatin (50 µM) ile muamele edildi. Gossypin'in hücre canlılığı ve etkili doz aralığı (5-100 µg/ml), 24, 48 ve 72. saatlerde MTT ile belirlendi. RNA izolasyonu ve cDNA sentezinden sonra, HSP60 ve HSP70 gen ekpresyon seviyesi RT-PCR ile analiz edildi. Gen ekspresyonu için $2^{-\Delta\Delta ct}$ methodu kullanıldı.
Bulgular: MTT sonuçlarına göre kanser hücre hatlarında 25-50-100 µg/ml gossipin dozlarının HSP60 ve HSP70 gen ekspresyon seviyeleri üzerinde etkili olduğu bulundu. Gossypin, üç hücre hattında HSP60 ve HSP70'in ekspresyonunu doza bağımlı olarak etkilemiştir. Üç hücre hattında, 50 µg/ml ve 100 µg/ml gossipin dozları, HSP60 ve HSP70'in ekspresyonunu kontrol grubuna kıyasla önemli ölçüde azalttı.
Sonuç: Sonuçlarımız, farklı hücre dizilerinde çeşitli dozlarda gossypinin antikarsinojenik etkisini güçlü bir şekilde desteklemektedir. Fakat, daha fazla in vivo araştırma ve insan çalışmalarına ihtiyaç olduğuna inanıyoruz. Bulgularımız, gossypin'nin farklı kanser türlerinin önlenmesi ve/veya tedavisi için yeni stratejiler geliştirmek için daha ileri araştırmalar için uygun aday ajan olabileceğini düşündürmektedir.
Anahtar kelimeler: Gossypin, HSP60, HSP70, MCF7, PC3, Hep3b

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INTRODUCTION

Flavonoids are reported to have a variety of anticancer effects. In particular, flavonoids modulate the activities of enzymes that scavenge reactive oxygen species (ROS) are involved in cell cycle arrest, apoptosis, autophagy, and suppression of proliferation and invasiveness of cancer cells[1]. The various species of the genus Hibiscus are used throughout the world as traditional medicines. Hibiscus is used to cure various ailments such as hypertension, cardiac diseases, stomachache, urinary problems, skin diseases, and many more[2]. Gossypin is known as a flavonol glucoside. It is found in the flowers of various plants such as *G. indicum*, *H. esculentus* and *H. vitifolius*[3-5]. Gossypin has antioxidant, anti-inflammatory[6,7], anticonvulsant[8], and anti-cancer effects[9,10]. Babu et al. (2003) reported that Gossypin reduced cell proliferation in various tumor cell lines (L929, HT29 and K562)[9]. Gossypin acted by activating apoptosis and inhibiting NFκB in Hep-3B cells[11]. But, the exact mechanisms of the anti-inflammatory and anti-carcinogenic activities of gossypin have not been fully elucidated[12].

Cells produce HSPs (chaperones) in response to thermal and other proteotoxic stresses and they have a critical role in the metabolism of most cancers[13]. HSP family members are classified according to their role in the immune system and consists of several subtypes based on molecular weight (HSP70, HSP90, HSP40, HSP60, etc.)[13]. HSPs are also involved in numerous processes in the cell, such as protein assembly, refolding of misfolded proteins and protein degradation[14,15]. HSPs have been found to be overexpressed in several cancers (lung, breast, and prostate)[16-19]. HSPs play an important role in oncogenesis and malignancy progression, as they can modulate all six hallmarks of cancer[20]. Overexpression of molecular chaperones has been associated with tumor survival, metastasis, and drug resistance of malignancies[21].

In the light of this information, we purposed to determine the effects of gossypin flavonide on HSPs genes in different cell lines including human prostate cancer (PC3), breast cancer (MCF7), and hepatoma (Hep3B).

MATERIALS AND METHODS

We optimized the cell culture experiments and IC_{50} values in our previous study[22]. PC3, MCF7 and Hep-3B cell lines were purchased from ATCC (USA). The cell lines in the Cryotube in the tank containing liquid nitrogen were removed and incubated in a water bath at 37°C for dissolve. The solubilized cells were transferred to T75 cm^2 flasks. After forty-eight hours, plates of three cell lines were counted at $2x10^5$ cells/well in DMEM containing 10% FBS. Then, seeded in a 96-well plate and incubated at 37°C in a humid atmosphere containing 5% CO_2.

After twentyfour hours, cells were exposed to different concentrations (5-100 μg/ml) of gosyypin (purchased from Sigma Aldrich) and cisplatin (50μM) as a positive control. Subsequently, a tetrazole MTT approach was performed with the cells at twenty-four, forty-eight and seventy-two hours, after which the absorbance value was assessed three times at 570 nm using a microplate reading spectrophotometer (Epoch Microplate Spectrophotometer, BioTek, USA). Viability rates were analyzed by comparison with control wells.

HSP60 and HSP70 gene expression analysis

Cells were seeded in 6-well plates with $2x10^5$ cells/well and incubated at 37°C in a humidified environment with 5% CO_2. After 6 hours of gossypin treatment, cells were removed from the 6-well plates by trypsinization and homogenized in TissueLyser II (Qiagen). RNA extraction was performed with the QIAcube RNA (Qiagen) isolation device according to by the manufacturer's advices.

The iScript™ cDNA Synthesis Kit (Bio-Rad) was preferred for the cDNA synthesis reaction. All cDNA synthesis processes were performed in the Medical Biology and Genetics Laboratory of Bayburt University. 4 μl of (5x) iScript reaction mixture, 1 μl of iScript reverse transcriptase, 8 μl of RNA (100 fg-1 μg) and furthermore 7 μl of water were added. The programme (priming 5 min at 25^0 C, reverse transcription 20 min at 46^0 C, RT inactivation 1 min at 95^0 C, optional step hold at 4^0C) was set in the thermocycler device (Sensequest-Labcycler) according to manufacture instructions. The cDNA samples were stored at -80 °C until the RT-PCR gene expression experiments.

The SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad) kit was used for mRNA expression processes. The SYBR Green kit contains antibody-mediated hot-start Sso7d fusion polymerase, dNTPs, $MgCl_2$, SYBR® Green I Dye, enhancers, stabilizers and a blend of passive reference dyes (including ROX and fluorescein). mRNA

expression processes were performed on the Bio-Rad CFX-96 RT-PCR instrument.

For mRNA expression experiments, 10 µl of SYBR mix (2X), 5 µl of cDNA (100 ng-100 fg), 1 µl of forwarding primer (10 µM), 1 µl of reverse primer (10 µM), and 3 µl of PCR-grade water were used per reaction. The PCR program (30 secs at 95^0C for activation, 5-15 sec at 95^0C for denaturation, and 15-30 sec at 60^0C for annealing (35-40 cycles)) was set according to company's suggestion. Beta-actin was chosen as the housekeeping gene. The $2^{-\Delta\Delta Ct}$ method was used for relative expression analysis of the HSP60 and HSP70 genes at the end of the RT-PCR reaction. To determine the expression of the genes, all evaluations were made by normalization with Beta-actin.

The preferred primer sequences for Beta-actin, HSP60 and HSP70 genes in this study are listed below;

Beta-actin;

F: GCTCCTGAGCGCAAGTACT

R: CGCTTGCTGATCCACATCT

HSP60;

F: CCAATGCTCACCGTAAGCC

R: CCTTGACTGCCACAACCTG

HSP70;

F: CTTCGACGTGTCCATCCTG

R: CTCCACGAAGTGGTTCACC

Statistical analysis

All data were analyzed using the package programs GraphPad Prism 7.04. D'Agostino-Pearson and Shapiro-Wilk normalization tests were performed to determine the distributions of the data. Since the groups in our study were independent samples with a normal distribution, the One-Way ANOVA test was performed to analyze the significance among groups. $P<0.05$ was regarded as significant.

RESULTS

According to MTT results of our previous study [22], IC_{50} value was determined using 5-100 µg/ml of gossypin doses. Three gossypin doses were designated to treat the cells (25, 50 and 100 µg/ml). In this study, effects of gossypin at concentrations of 25, 50, and 100 µg/ml on gene expressions levels of HSP60 and HSP70 were analyzed. The expression levels of HSP60 and HSP70 genes decreased with increasing gossypin concentrations. HSP60 also demonstrated significantly reduced gene expression level in PC3 cells at concentrations 50 µg/ml and 100 µg/ml compare to control group ($p=0.0077$; $p<0.0001$; respectively) (Figure 1A). 100 µg/ml gossypin concentration showed stronger effect than others.

In particular, gossypin concentrations at 50 µg/ml and 100 µg/ml diminished HSP70 gene expression compare to control group in PC3 cells ($p=0.0004$; $p<0.0001$; respectively) (Figure 1B).

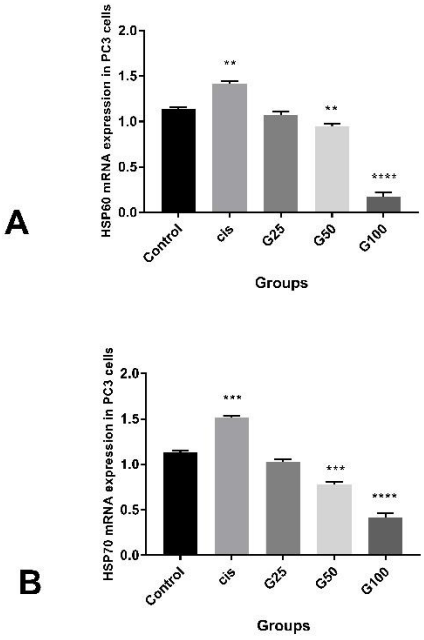

Figure 1. A) HSP60 mRNA expression level in PC3 cells B) HSP70 mRNA expression in PC3 cells

According to our findings, in MCF7 cells 25 µg/ml, 50 µg/ml and 100 µg/ml concentrations of gossypin caused to decline of HSP60 expression significantly ($p=0.0482$; $p=0.0131$; $p=0.0012$; respectively) (Figure 2A). Moreover, 100 µg/ml was the most effective compare to the other gossypin concentrations. Expression of HSP70 at 50 µg/ml and 100 µg/ml of gossypin concentration was decreased compare to control group ($p=0.0104$; $p=0.0027$; respectively) (Figure 2B). 25 µg/ml of gossypin dose did not change the HSP70 expression significantly ($p=0.4088$).

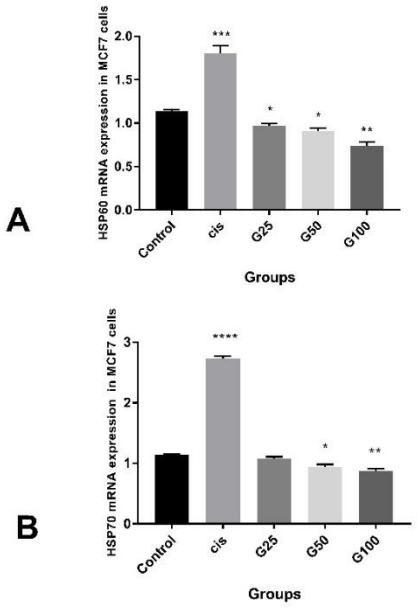

Figure 2. A) HSP60 mRNA expression in MCF7 cells B) HSP70 mRNA expression in MCF7 cells

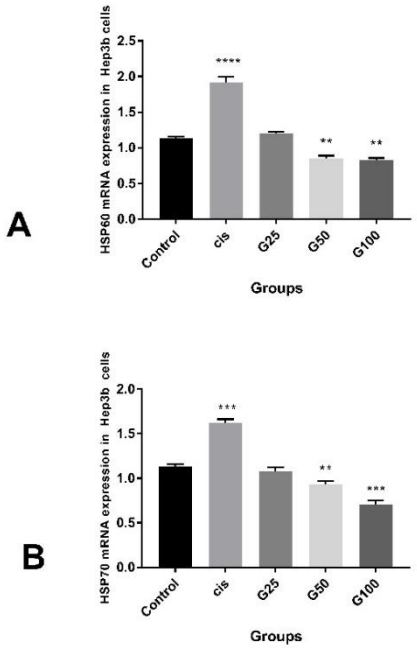

Figure 3. A) HSP60 mRNA expression in Hep3b cells B) HSP70 mRNA expression in Hep3b cells

Hep3b cells treated with 50 μg/ml and 100 μg/ml gossypin concentrations demonstrated lower HSP60 gene expression than control group (p=0.0031; p= 0.0021; respectively) (Figure 3A). As shown in Figure 3B, HSP70 gene expression was lower in Hep3b cells treated with was 50 μg/ml and 100 μg/ml gossypin concentrations than the control group (p=0.0093; p=0.0003; respectively)

DISCUSSION

Many people suffer from breast, prostate and hepatocellular carcinomas. Considering the many side effects of chemotherapy in cancer treatment, we can say that the demand for treatment methods using natural personal products is increasing day by day. Anti-carcinogenic effects of gossypin was demonstrated in published studies. Furthermore, HSPs have active roles in cancer development and progression. Although presented studies no one of them demonstrates influence of gossypin on HSP60 and HSP70. In this research, we examined the effects of gossypin on gene expression of HSPs in three types of cancer cell lines. Therefore, we believe that this study may be valuable as it is the first in the literature.

HSPs have been linked to cellular responses to various types of extrinsic stress (heat, heavy metals or oxidative stress exposure). HSPs play an important role in cell homeostasis and apoptosis regulation [23]. Overexpression of HSPs is found in various cancers (breast, prostate, colon, etc.)[24]. Many HSPs contribute to metastatic phenotype of tumors[25]. Therefore, HSPs are involved in the pathogenesis, diagnosis, prognosis, and treatment of many cancers[26]. In prostate cancer (PCa), certain chaperone members such as HSP90, HSP70, HSP27, and clustering are vital regulators of androgen receptor (AR) folding and trafficking[27]. Several studies have reported the effect of HSP60 on the development of PCa[28,29]. HSP60, HSP70 and HSP90 were upregulated in human hepatocellular carcinoma cells in a stress-specific manner[30].

Herbal and nutritional supplements have always been used to treat cancer[31]. These compounds may also be useful in cancer prevention and treatment. Gossypin, a flavone has been found to inhibit angiogenesis, inflammation, and carcinogenesis. However, the mechanisms of these activities are mysterious[12]. Gossypin has been shown to have antioxidant, and anticarcinogenic activity[9].

Gossypin reduced S phase and caused to cell cycle arrest in G2/M phase in gastric cancer[32]. Moreover, gossypin induced intrinsic apoptosis. Gossypin showed greater cytotoxicity in melanoma cells with BRAFV600E mutation than wild type[33]. In another study, gossypin stimulated apoptosis in myeloma cells activating the apoptotic caspase-3/7 pathway[12]. Gossypin can inhibit NF-κB activation induced by inflammatory stimuli[12]. In one study, it was observed that treatment of U251 cells with gossypin inhibited cell proliferation in a dose and time-dependent manner and it was minimally toxic to normal human astrocytes[34]. Gossypin suppressed MMP-9 expression and TNF-stimulated invasive activity of H1299 cells. Other studies showed that gossypin blocked the development of primary tumors and the progression of malignancies[9,12]. Cinar (2021) showed that anti-proliferative and apoptotic effects of gossypin on PC3 cells[22]. Moreover, Cinar (2021) showed that caspase-3 and caspase-9 levels were increased with gossypin application. Therefore, gossypin triggers apoptosis[22]. In this study we showed that three gossypin doses (25, 50 and 100 μg/ml) may be anti-cancer effects on PC3, MCF7 and Hep3b cells.

We found no studies showed effects of gossypin on HSPs expression in cancer. However, there are several studies investigating the effects of various flavonoids on HSPs. Exposure of HepG2 cells to scutellarin 14b resulted in a dramatic decrease in the levels of Hsp27, Hsp 60, Hsp 70, HO1 and HO2[35]. Sahin et al. (2009) showed that although Hsp70 levels were lower in the genistein groups than in the control group, the expression of Hsp60 and Hsp70 did not differ between groups[36]. Quercetin effectively suppressed the expression of Hsp27, Hsp70 and Hsp90 in breast cancer[37]. Hassanzadeh et al. (2019) found decrease level of HSP70 expressions when human chronic myeloid leukemia K562 cells were treated with quercetin[38]. In MDA-MB-231 and MDA-MB-468 BCa cells, TL-2-8, quercetin treatment caused degradation of several Hsp90 client proteins without inducing Hsp70[39]. The expression of survivin and HSP70 proteins were downregulated in SKOV-3 cells treated with artonin E[40]. In the study used HCT-116 cell line, cells were subjected to heat shock in the presence of fisetin and the induction of HSF1 target proteins, such as HSP70 and HSP27, was inhibited[41].

In this study, the effects of 25, 50 and 100 μg/ml concentration of gossypin on the expression of HSP60 and HSP70 in different cell lines were investigated using the method of RT-PCR. Similar to the above studies showed the effects of different flavonides on HSP in cancer cells, we revealed that the 50 μg/ml and 100 μg/ml gossypin concentrations decreased HSP60 and HSP70 gene expression in PC3, MCF7 and Hep3b cell lines to different degrees. We believe that, gossypin usage may contribute to decrease HSP60 and HSP70 expression in patients with high level HSPs.

Although this study is the first to explore the effects of gossypin on HSPs in prostate, breast and hepatoma cancer cells, it has several limitations. First, we investigated effects of gossypin in just three cancer cell lines. However, studies with other cancer lines may contribute to further understanding of gossypin effects on HSPs. Second, we investigated effects of gossypin on just HSP60 and HSP70. Exploring effects of gossypin on other HPSs will contribute to clarify mechanism of cancer development.

In conclusion, gossypin decreased the expression of HSP60 and HSP70 genes in prostate, breast cancer and hepatoma cancer cells. Following the results of the study and the use of gossypin, further studies are recommended to investigate the effects of this anti-cancer agent on different types of cancer and other genes.

Yazar Katkıları: Çalışma konsepti/Tasarımı: ED, İÇ; Veri toplama: ED, İÇ; Veri analizi ve yorumlama: ED, İÇ; Yazı taslağı: ED, İÇ; İçeriğin eleştirel incelenmesi: ED, İÇ; Son onay ve sorumluluk: ED, İÇ; Teknik ve malzeme desteği: ED, İÇ; Süpervizyon: ED, İÇ; Fon sağlama (mevcut ise): yok.
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