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EFFECT OF HEPARIN ON HUMAN SKIN FIBROBLAST PROLIFERATION IN VITRO

IN VITRODA HEPARİNİN İNSAN DERİ FİBROBLASTLARININ ÇOĞALMASI ÜZERİNDEKİ ETKİSİ

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Anahtar Sözcükler : Heparin, sitotoksisite, deri fibroblastı, MTT

Key Words: Heparin, cytotoxicity, skin fibroblast, MTT

SUMMARY

The dose-related effects of standard heparin on the proliferation of cultured human skin fibroblasts were investigated in the cell cultures which were established using human skin biopsy material. It was determined that the high concentrations [1/2 -1/64, (2500-78 IU/ml)] of standard heparin was cytotoxic. In low concentrations [1/128-1/1024, (39-5 IU/ml)], heparin showed an effect in proliferation way. The correlation between heparin concentration and the proliferation of cultured human skin fibroblasts was found to be 90 % and this result is statistically significant ($p<0.001$, $r=0.9498$). These results are discussed with the concerned literatures.

ÖZET

Standart heparinin, kültüre edilmiş insan deri fibroblastlarının çoğalması üzerindeki doza bağlı etkisi, insan deri biyopsi materyalinin kullanımı amacıyla oluşturulan hücre kültürlerinde araştırılmıştır. Standart heparinin yüksek konsantrasyonlarının [1/2 – 1/64, (2500-78 IU/ml)] sitotoksik olduğu belirlenmiştir. Düşük konsantrasyonlarda [1/128 – 1/1024, (39-5 IU/ml)] ise heparin, hücrelerin çöğalma yolunda bir etki göstermiştir. Heparin konsantrasyonu ve kültüre edilmiş insan deri fibroblastları arasındaki ilişki %90 olarak bulunmuştur ve bu sonuç istatistiksel olarak oldukça önemlidir ($p<0.001$, $r=0.9498$). Bu sonuçlar ilgili literatürlerde tartışılmıştır.

INTRODUCTION

Heparin (HP) is a glycosaminoglycan with repeating sequences of D-S- glucuronic acid-1,4-α-S-glucuronic acid-1,4-α-S-NacGlc. HP is a complex polysaccharide covalently linked to several proteins' core and effect to their molecules activity. Besides its well-known action as an anticoagulant, antiproliferative activity of HP has been demonstrated for various cell types (1, 2).

Heparin stimulates endotelial cell growth while it inhibits the proliferation of renal mesangial cells, rat cervical epithelial cells, transformed cell lines and systemic smooth muscle cells (SMCs) and pulmonary artery smooth muscle cells (PASMCs) (1-7).

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Heparin blocks the cell cycle either at the Go/G1 transition point or at mid to late G1 progression and may inhibit such cellular intermediate processes as protein kinase C activation, c-Fos and c-Myc induction, activator protein-1/Fos-Jun binding, and posttransitional modification of Jun B. Heparin has also been shown to selectively block the protein kinase C pathway of mitogenic signaling and the phosphorylation of mitogen-activated protein kinase (1, 2).

Fibroblasts are multipotent cells of connective tissue, whick they can give rise to other cells of mesodermal origin such as fat cells, bone cells, cartilage cells and smooth muscle cells. These cells synthesis and secrete extracellular matrix and collagen to repair tissue damage (8). Proliferation of fibroblasts is a serious problem in surgical wound healing. Depending on the location of the

injury, the growth factor of fibroblasts can lead to different problems (3).

In our study, we aimed to investigate the effect of various dilutions (1/2- 1/1024) of standard heparin (5000 IU/ml) on the proliferation of cultured human skin fibroblasts (HSF).

MATERIALS AND METHODS

Cell Culture:

Fibroblasts were obtained from human skin biopsy and were cultured by using DMEM with 10% FCS supplemented with Penicillin-Streptomycin (100Uml / 100 µg/ml) and 2Mm L-Glutamine and cultures were incubated at 37^0C in a humidified atmosphere of 5% CO_2 and 95% air. The media were replaced every 2 or 3 day until confluence was reached. Fibroblasts used for this study were between their 3th and 5th passage in culture.

MTT Assay:

Cell survival was analyzed by using a nonradioactive cell proliferation assay system (MTT assay) consisting of 3-(4,5-dimethylthiaziazol-2-yl) 2,5-diphenyl tetrazolium bromide

The cells were plated at $2x10^5$ cell/ml per well into 96 well plates in various concentrations of heparin [1/2-1/64, (2500-78IU/ ml)] and [1/128-1/1024, (39-5 IU/ml)] were added with serum-free DMEM in a total volume of 100 µl/well. After further culture for 24 hours at 37^0C in a humidified atmosphere of 5% CO_2 in air, fibroblast growth-induction activity was determined by the MTT solution (5mg/ml) which was added to each well of the plate and plates were incubated for 4 hours at 37 ^{0}C in a humidified atmosphere of 5% CO_2 in air. The medium in each well was changed by 100 µl dimethylsulfoxide (DMSO) and mixed thoroughly to dissolve the dark blue crystals for 10 minutes at room temperature in order to ensure all crystals could be dissolved. The plates were read with ELISA reader (EL X 808), using test wavelength of 570 nm and a reference wavelength of 630nm. All assays were repeated two times.

Cytotoxic response;

% Viability= $\frac{a - b}{c - b}$ is calculated with this formula.

a: The average OD570 value of the test groups' well

b: The average OD570 value of the blank well

c: The average OD570 value of the control groups' well

The results were compared statistically by evaluating regression analysis in SPSS for windows.

RESULTS

The cells were examined in phase-contrast microscopy and were determined morphologically by cell proliferation and MTT assay. In microscopic examination, low concentrations [1/128-1/1024, (39-5 IU/ml)] of heparin stimulated fibroblast cell growth in presence of fetal bovine serum (FBS). In MTT assay; low concentrations [1/128-1/1024, (39-5 IU/ml)] of standard heparin showed a stimulation of cell proliferation, but high concentrations which was cytotoxic [½ - 1/64, (2500-78 IU/ml)] inhibited the proliferation. The concentration of heparin that inhibited growth by 50% (IC_{50}) was found 2500-1250 IU/ml (1/2-1/4) (fig. 1).

The correlation between heparin concentration and proliferation was found 90% which was statistically significant ($p< 0.001$, $R=0.94971$).

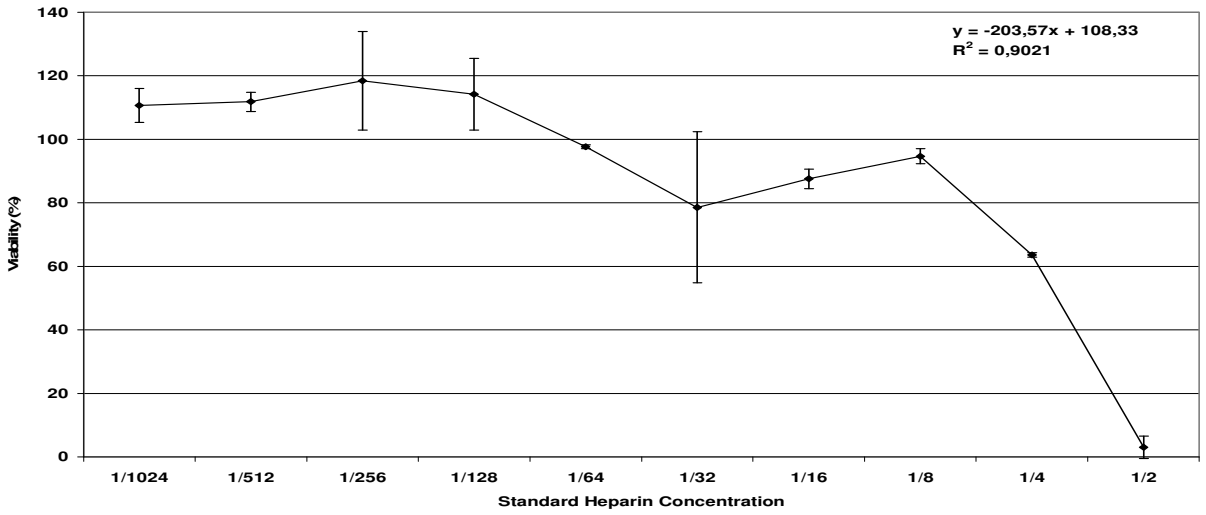

Figure 1. Relationship between Heparin concentration and cell viability

DISCUSSION

The aim of the present investigation was to determine the effect of heparin on proliferation of cultured HSF. Anti-proliferative activity of heparin has been demonstrated in various cell types, including mesangial cells, smooth muscle cells, lens epithelial cells and scleral fibroblasts (1).

Heparin showed a preventive effect on proliferation in the endothelial cells (200-500 µg /ml) and scleral fibroblasts (60µg /ml) at higher concentrations, however it showed the same effect at lower concentration rates (2 and 5 µg / ml) in smooth muscle and mesangial cells. The investigation of cell culture morphologically made by MTT assay demonstrated that the high concentrations [1/2-1/64, (2500-78 IU/ml)] of standard heparin was cytotoxic and inhibited the cell proliferation (3, 9).

Regarding the effect of heparin on fibroblasts in monolayer culture, heparin stimulated the fibroblast growth with an optimal response at 0.01mg/ml and the volume of the treated fibroblasts was smaller than that of untreated controls (4). In the another study, heparin stimulated the proliferation of normal human lung fibroblast at low concentrations (0.03, 0.03-1.0 µg / ml) and it was observed that higher concentration (100 µg / ml) had an inhibitory effect (10).

Denk et al. investigated the effect of heparin on cultured human corneal fibroblasts (HCF) proliferation with and without growth factors. Ten percent of fetal calf serum was used to determine the effect of heparin on the proliferation of HCF in concentrations ranging from 12.5 µg / ml to 5000 µg / ml, and heparin inhibited the proliferation of HCF significantly (1).

Johnson et al. indicated that heparin (1000 U/ml) effects the DNA synthesis and inhibits the cell proliferation in smooth muscle cells of human respiratory system (5). The mechanism of the anti-proliferative effect of heparin has not been completely understood to date. Previous studies have demonstrated that heparin binds to cell surface and it is internalized by an endocytotic mechanism (11, 12). In our study high concentration of heparin showed anti-proliferative effect. There have been several studies that heparin has inhibited cell cycle in endothelial and smooth muscle cells and it is reported that this effect is occured by inhibition of cyclin-dependent kinase (CDK) (13,14,15). On the other hand there is no study about the inhibition of CDK in HSF in the literature. It has been also reported that heparin inhibits cell cycle due to its possible cytotoxic effect. Since heparin blocked polymerase chain reaction, it may be speculated that heparin may block the human DNA polymerase or subunit of it which is the proliferating cell nuclear antigen (PCNA).

Results sourced from the other studies have also suggested that the antiproliferative effect of heparin results from a modulation of transmembrane signal events. It has also been demonstrated that heparin antagonizes the inositol 1, 4, 5-trisphosphate – activated calcium release in a dose- dependent manner (16, 17) and that heparin selectively inhibits protein kinase C-dependent mitogenic signaling pathways, such as induction or transcription of several genes associated with cell cycle progression (18-20).

In conclusion, the results of this study indicate that heparin has a stimulating effect on skin fibroblast proliferation at low concentrations, but an inhibition and a cytotoxic effect at high concentrations. Heparin may be a valuable therapeutic agent for the prevention of fibroblast proliferation such as scar formation when used at an appropriate concentration.

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