

## EXPERIMENTAL STUDY

## The effects of heparin on DLD-1 colon cancer cell line

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**Abstract:** *Background:* Patients with cancer are frequently treated with anticoagulants, including heparin, to prevent or to treat the deep vein thrombosis. It has been indicated that heparin affects the survival of patients with cancer. Also, the apoptotic and antiproliferative effects of heparin on some cancers has been demonstrated. Experimental studies support the hypothesis that cancer progression can be influenced by heparin, but the results of these studies are not conclusive.

*Objectives:* We planned to investigate the effects of different concentrations of heparin in the colon cancer cell line DLD-1. *Methods:* This study was done by the addition of heparin in different doses into colon cancer cell line DLD-1 in vitro. After an incubation period of 72 hours, study and control groups were evaluated for viable cell count, percentage of proliferating index and apoptosis percentage.

*Results:* The result of the viable cell count in the second and third study groups ( $98.35 \pm 27.3$ ,  $97.23 \pm 39.38$ ) were low compared to the control group. The results of the proliferative index of study groups ( $46.47 \pm 10.44$ ,  $47.23 \pm 12.03$ ,  $45.55 \pm 14.2$ ) were higher than the control group ( $40.62 \pm 9.28$ ). The results of apoptosis in study groups ( $14.35 \pm 1.93$ ,  $16.47 \pm 7.25$ ,  $13.56 \pm 5.66$ ) were lower compared to the control group ( $22.17 \pm 15.9$ ). But these results were not statistically significant.

*Conclusion:* Heparin has no significant anti-proliferative and apoptotic effects on colon cancer cells in vitro. Therefore, for clinical applications, more advanced studies are needed to examine the effect of heparin on colon cancer (Tab. 3, Fig. 3, Ref. 25). Full Text (Free, PDF) [www.bmj.sk](http://www.bmj.sk).

Key words: heparin, colon cancer, DLD-1 cell line.

Some drugs used in cancer treatment lead to thromboembolic events by causing changes in blood stream, endothelial cell injury and release of procoagulants (1). Anticoagulant agents are thus added to treatment regimens of many cancer patients. The finding of a longer survival in cancer patients treated with heparin for deep vein thrombosis has formed the hypothesis that heparin may have effects on cancer progression (2). Many studies evaluating the effects of heparin on tumor growth and metastases has been done but results are not conclusive and satisfactory (3).

Besides its anticoagulant effects, heparin has antihypertensive, antiinflammatory and antiproliferative effects (4-8). It is also shown that it has decreasing effects on tumor angiogenesis and increasing effects on apoptosis of neutrophils, lymphoblasts and mononuclear cells (9, 10). The apoptotic and antiproliferative effects of heparin have been shown in nasopharyngeal carcinoma and hepatoma cell lines (11-13). There are some studies evalu-

ating the effects of heparin on tumor growth and metastases in colon cancer cell lines but there is no in vitro study showing its apoptotic effects in the literature (14, 15).

The aim of this study is to assess the effects of heparin on cell cycle together with its apoptotic and antiproliferative effects in colon cancer cell lines.

### Materials and methods

#### *The preparation of DLD-1 (ATCC, CCL-221) human colon adenocarcinoma cell lines*

It was an in vitro study performed on human colon cancer cell line DLD-1 (ATCC) (American type Culture Collection-CCL-221). The cells have been isolated from male cancer patients with 0 blood group and Dukes C tumor in 1979 and have c-myc, myb, ras, (H,K,N), fos, sis, p53 and DNA repair gene mutations (16). Before the experiment, the cells kept at -80 °C were left to dissolve at room temperature. RPMI 1640 medium composed of 10% fetal calf serum (FCS) (Biochrom KG, S 0113), 100 units/ml of penicillin (Llorente Laboratories, Madrid, Spain) and 100 µg/ml of streptomycin (Llorente Laboratories, Madrid, Spain) was added to the cells transferred to conic tube and centrifuged at 800 G for 10 minutes. The supernatant was removed from the medium and the precipitated cells were homogenized with RPMI 1640 medium. This assay was repeated three times. The cells gained by this way were taken into 25 ml culture flasks.

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**Tab. 1. Rates of cell viability.**

Groups	Mean±SD	p value <0.05
Control	0.123±0.57	–
Group I (50U/L heparin)	0.128±0.55	NS
Group II (100U/L heparin)	0.115±0.50	NS
Group III (200U/L heparin)	0.120±0.85	NS

NS – not significant

The cells (DLD-1) were incubated at 37 °C in 5 % CO<sub>2</sub> containing medium until they become 75 % confluent at the bottom of the culture flask. Once in three days new culture medium was added. After they become 75 % confluent at the bottom of the culture flask, trypsinisation procedure was applied to cultured cells to separate them from the flask (17). Before this procedure, non-adherent cells and medium were removed from the flask. Adhered cells were washed twice with PBS, then 5 ml of sterile 0.25 % trypsin was added to the flask and incubated for 5 minutes at 37 °C in 5 % CO<sub>2</sub> containing medium. Five minutes later flasks were removed from the incubator and examined under an inverted microscope to make sure that adhered cells were separated. Then FCS was added to inactivate trypsin. The cell suspension obtained by adding RPMI 1640 medium was taken into conic tube and centrifuged at 800 G for 10 minutes. Following centrifugation, the supernatant was removed and the cells precipitated at the bottom of conic tube were washed with culture medium to make them ready for usage.

*Cell culture and study groups*

Human colon adenocarcinoma cells were cultured in 96 well plates (10<sup>5</sup>/200 µL), in RPMI 1640 and then after being divided into 4 groups; the following doses of heparin (Sigma, H 3393) were added into the wells after being dissolved in RPMI 1640. Cultured human colon adenocarcinoma cells without medicine were used as a control.

Group I: Control

Group II: 50 U/mL heparin

Group III: 100 U/mL heparin

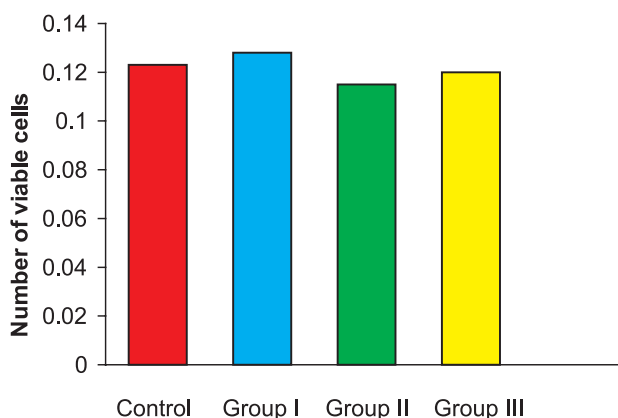
Group IV: 200 U/mL heparin

Then the culture plates were left for incubation at 37 °C in 5 % CO<sub>2</sub> containing medium for 72 hours.

**Analysis of results**

*Viable cell ratio:* Viable cell ratio in each study group was obtained by MTT test, which shows the amount of mitochondrial activated cells in cell culture (17). The results of the test determined were with a microelisa calculator (Anthos labtec instruments) at 540–620 nm.

*Apoptosis and proliferation:* The Coulter DNA-prep reagent system (Miami FL, USA) was used to stain the DNA of the cultured cells with propidium iodide (PI) for the quantitative measurement of cellular deoxyribonucleic acid (DNA) content by flow cytometry with the Coulter DNA Prep workstation (Florida USA). Flow cytometry was performed on Coulter Ep-



**Fig. 1. Comparison of viable cell ratio in each group.**

ics Elite flow cytometry (Florida USA). Data were analysed for apoptosis and cell cycle using the Multicycle AV Software (Phoenix Flow System, San Diego, CA). Apoptosis ratio of cultured mononuclear cells was measured as a percentage of the hipodiploidic peak. The proliferation ratio of cultured cells was assessed using the formula shown below. Proliferative index (%) = 100 x (cells in mitosis + cells in S-phase) / total cell number

*Statistical analysis*

Viable cell ratios were evaluated by the Student t test. The data were presented as arithmetic mean ± standard deviation. p values less than 0.05 were accepted statistically significant.

In the assessment of the data in terms of proliferative index and apoptosis, the Mann–Whitney U test was used. p values less than 0.05 were accepted statistically significant.

**Results**

In this study, the viable cell ratio, proliferative index and percentage of apoptosis were determined in each group.

*Viable cell ratio:* Control groups and study groups were compared in terms of their viable cell number. Values measured at 540 nm at microelisa reader with MTT method are given at Table 1 and Figure 1. The viable cell ratio was 108.06±26.05 in the group 1, 98.35±27.30 in the group 2 and 97.23±39.38 in the group 3. Compared to the control group, the values were higher in the group 1 and lower in the other two groups. But these differences were not statistically significant (Tab. 1, Fig. 1).

**Tab. 2. Proliferative index (%).**

Groups	Proliferative index Mean±SD	p value <0.05
Control	40.62±9.28	–
Group I (50U/L heparin)	46.47±10.44	NS
Group II (100U/L heparin)	47.23±12.03	NS
GroupIII(200U/L heparin)	45.55±14.42	NS

NS – not significant

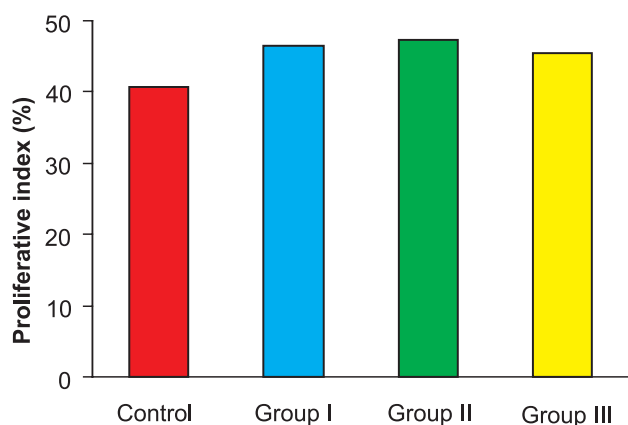


Fig. 2. Comparison of proliferation index (%) in each group.

**Proliferation index:** The control group and other groups were compared in terms of their proliferation indices. The proliferation index was found  $40.62 \pm 9.28$  in the control group,  $46.47 \pm 10.44$  in the group 1,  $47.23 \pm 12.03$  in the group 2 and  $45.55 \pm 14.42$  in the group 3. Although the values found in groups 1, 2 and 3 were higher than the values found in the control group, this difference was not statistically significant (Tab. 2, Fig. 2).

**Apoptosis:** The control group and other groups were compared in terms of their apoptosis percentage. The percentage of apoptotic peak were following:  $22.17 \pm 15.9$  in the control group,  $14.35 \pm 11.93$  in the group 1,  $16.47 \pm 7.25$  in the group 2,  $13.56 \pm 5.66$  in the group 3. Despite the fact, that the percentage of apoptotic peak was lower in all groups compared to the control group, this difference was not statistically significant (Tab. 3, Fig. 3).

## Discussion

While in some experimental studies heparin has been shown to have an inhibitor effect on tumor growth, in some others it has been shown to have no effect (11–14). These two different results are related to differences in study design and the presence of different clones that could not be inhibited by heparin in tumor cell population (18).

There is not much data about the role of heparin in the apoptotic process. However, it is known that while heparin increases apoptosis in human neutrophile, lymphoblast and mononuclear cells, on the other hand it inhibits apoptosis in mesangial culture cells and glomerular cells (10, 19).

In our study, it was determined that heparin did not have any effect on apoptosis on cell line CCL-221. Although heparin decreased apoptosis in the study groups compared to controls, this was statistically not significant. Biochemical marker of apoptosis is internucleosomal DNA fragmentation (10). Wright et al showed that heparin binds to the 24 kD protease which activates DNA fragmentation in cells going to apoptosis (20). Thus in some experimental models it is thought that inhibition of this enzyme with heparin suppresses apoptosis. Maeda et al showed that

Tab. 3. Apoptosis (%).

Groups	Apoptosis Mean±SD	p value <0.05
Control	22.17±15.9	–
Group 1 (50U/L heparin)	14.35±11.93	NS
Group 2 (100U/L heparin)	16.47±7.25	NS
Group 3 (200U/L heparin)	13.56±5.66	NS

NS – not significant

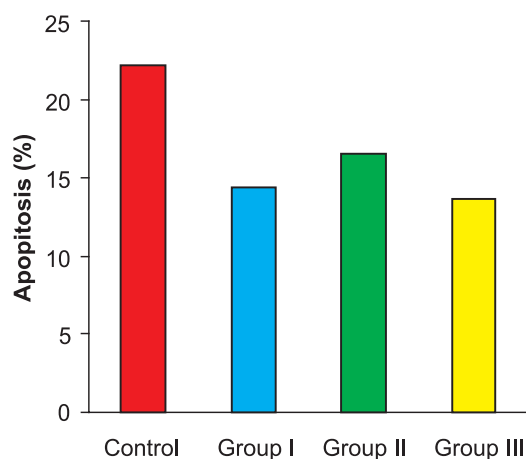


Fig. 3. Comparison of apoptosis percentage in each group.

internucleosomal DNA fragmentation as a marker of apoptosis could be induced in rat hepatocyte culture depending on the cell concentration (21). It was also observed that their viability had varied according to the concentration of DNA fragmentation in cells. In this study, with the inhibition of the enzyme, which activates DNA fragmentation, it was found that heparin depresses DNA fragmentation and prevents cell death to some degree (21). The reason why apoptosis can not take place in cell line CCL-221 in the presence of heparin may be explained by the inhibition of 24 kD protease mentioned above by heparin. Li et al showed that heparin induced apoptosis by increasing the expression of c-myc in their study in which they searched the apoptotic effects of heparin on nasopharynx cell line CNE2 (11). CCL-221 cell line used in our study expresses c-myc protooncogene (16). C-myc protooncogene has two challenging functions as increasing both apoptosis and cell proliferation at the same time (11). In our study no apoptotic effect of heparin but an increase in cell proliferation although not statistically significant may be explained by the proliferating property of c-myc protooncogene together with its apoptotic property (12, 16).

When the cell groups in our study were examined in terms of the proliferative index, it was seen that proliferation had increased in the study groups compared to the control group. However this increase was not statistically significant. In a study done by Fishman et al it was shown that heparin related disaccharides could stimulate or inhibit the cell proliferation in colon cancer cell lines KM12 and KM12SM by affecting the phosphorylation

of the erb-b receptor family found on the cell surface (22). The fact that the proliferation increased a bit compared to the control group, although not statistically significant, suggests the relation of heparin with cell surface receptors.

Although heparin has been shown to have many different effects in vitro, these effects have not been shown in vivo yet (23). For example, though heparin has been shown to have antimetastatic effects in experimental models, in clinical studies different results have been obtained (24).

Tumor growth depends on the balance between the stimulating and inhibiting factors and shows varied characteristics for each different tumor cell (11, 23). In our study, increasing doses of heparin were found to be ineffective in colon cancer cell line CCL-221 in terms of viable cell ratio, apoptosis and proliferation. This situation can be explained with the mechanisms mentioned above, like inhibition of the enzyme that activates DNA fragmentation or the presence of different culture cell concentration. There are few studies on this subject that evaluate in vitro effects of heparin on colon cancer cell lines. In the study performed by Anthacopoulos et al, heparin was shown to be ineffective on tumor growth (14). Negative results were obtained in clinical studies examining the effects of heparin treatment with chemotherapy or alone in patients with colorectal cancer (24, 25).

As a result, heparin did not show any apoptotic and antiproliferative effect on cell line DLD-1 in vitro. The effect of heparin in other colon cancer cell lines needs to be determined.

## References

1. **Aruffo A, Bevilacqua MP, Nelson RM, Cecconi O, Roberts WG, Linhardt RJ.** Heparin oligosaccharides bind L- and P-selectin and inhibit acute inflammation. *Blood* 1993; 82: 3253–3258.
2. **Collen A, Van Noorden CJF, Peters E, Lupu F, Koolwijk P, Smorenburg SM, Van Hinsberg VW.** The effects of unfractionated and low molecular weight heparins on microvascular endothelial cell proliferation and formation of capillary-like tubular structures in a fibrin matrix. *Cancer* 2000; 60: 6196–6200.
3. **Collen A, Noorden CV, Peters E et al.** Unfractionated and low molecular weight heparin affect fibrin structure and angiogenesis in vitro. *Cancer Res* 2000; 60: 6196–6200.
4. **Letai A, Kuter DJ.** Cancer, coagulation, and anticoagulation. *Oncologist* 1999; 4: 443–449.
5. **Obermair A, Bancher-Todesca D, Gitsch G, Bilgi S, Leodolter S.** Correlation of vascular endothelial growth factor expression and microvessel density in cervical intraepithelial neoplasia. *J Natl Cancer Inst* 1997; 89: 1212–1217.
6. **Ray A, Gouda I, Swedenburg J, Lundmark K et al.** Inhibition of rat smooth muscle cell adhesion and proliferation by non-anticoagulant heparins. *J Cell Physiol* 2002; 193: 35–72.
7. **Yıldırım A, Erduran E, Tekelioğlu T, Gedik Y.** Apoptotic effects of heparin on lymphoblasts, neutrophils, and mononuclear cells: results of a preliminary in vitro study. *Amer J Haematol* 1999; 61: 90–93.
8. **Xiong AH, Li HL, Zhang HW, Ye KH et al.** Effects of heparin on apoptosis in human nasopharyngeal carcinoma CNE2 cells. *Cell Res* 2001; 11: 311–315.
9. **Mandal AK, Saklayen MG, Layden TW.** Heparin lowers blood pressure: biological and clinical perspectives. *Kidney Int* 1995; 1017–1022.
10. **Fauci AS, Braunwald E et al.** Gastrointestinal tract cancer. 571–576. In: Braunwald E, Fauci AS (Eds). *Harrison's Principles of Internal Medicine*. New York; McGraw-Hill, 1998.
11. **Boeryd B, Hagmar B.** Disseminating effect of heparin on experimental tumour metastases. *Pat Europ* 1969; 4: 274–282.
12. **Cosmi B, Piovalla F, Hirsh J, Ginsberg JS, Siragusa S.** Low-molecular-weight heparins and unfractionated heparin in the treatment of patients with acute venous thromboembolism: results of a meta-analysis. *Ann J Med* 1996; 100: 269–277.
13. **Glimelius B, Pohlmen L.** Swedish rectal cancer trial. Improved survival with preoperative radiotherapy in resectable rectal cancer. *New Engl J Med* 1997; 336: 980–987.
14. **Gogly B, Pella B, Godeau G, Groult N, Hornebeck W.** Influence of heparin(s) on the interleukin-1-beta-induced expression of collagenase, stromelysin-1, and tissue inhibitor of metalloproteinase-1 in human gingival fibroblast. *Biochem Pharmacol* 1998; 56: 1447–1454.
15. **Coller BS, Beutler E et al.** Heparin, Hirudin and related agents. 1793–1796. In: Beutler E, Coller BS (Eds). *Williams Hematology*. New York; The McGraw-Hill, 2001.
16. **Yamazaki H, Oi H, Tang JT, Matsushita M, Inoue T, Nose T.** Heparin induces rapid and remarkable elevation of hepatocyte growth factor/scatter factor during trans arterial embolization in renal cell carcinoma. *Anticancer Res* 1997; 17: 1435–1437.
17. **Singer HA, Mishra-Gorur K, Castellot JJ Jr.** Heparin inhibits phosphorylation and autonomous activity of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II in vascular smooth muscle cells. *Amer J Pathol* 2002; 11: 1893–1901.
18. **Zvibel I, Papa M, Brill S, Fishman S, Halpern Z.** Heparin-derived disaccharides modulate proliferation and erb-b2 mediated signal transduction in colon cancer cell lines. *Int J Cancer* 2002; 99: 179–184.
19. **Talbot IC, Clark ML.** Tumours of the gastrointestinal tract. 1990–1993. In: Weatherall DJ, Ledingham GG, Warrell DA (Eds). *Williams Hematology: Oxford textbook of medicine*. New York; Oxford University Press, 1996.
20. **Zimmerman J, Bar-Shavit R, Ben-Sasson SA, Peretz T.** Control of cell proliferation by heparan sulfate and heparin binding growth factors. *Thromb Haemost* 1995; 74: 534–540.
21. **Grendell JH, McQuaid KR, Bresalier RS, Friedman SL.** Malignant and premalignant lesions of the colon. *Diagnosis & Treatment in Gastroenterology*. Prentice-Hall Int 1996; 367–391.
22. **Lo K, Fung LF, Yuen PW, Tsao SW, Wang XH, Liu Y.** Differential expression in nasopharyngeal carcinoma cells. *Life Sci* 2000; 67: 923–936.
23. **Arenberg DA, Glass M, Burdick MD, Polverini PJ, Streiter RM, Kunkel SL.** Inhibition of interleukin-8 reduces tumorigenesis of human non-small cell lung cancer in SCID mice. *J Clin Invest* 1996; 97: 2792–2802.
24. **Levin B, Goldman L et al.** Neoplasms of the large and small intestines. 741–749. In: Thomas EA, Bennett JC (Eds). *Cecil Textbook of Medicine*. New York; W.B. Saunders Company, 2000.
25. **Kayaalp SO.** Antikoagulan ilaçlar. 586–591. *Tibbi farmakoloji*. Ankara; Hacettepe-Tas Kitapçılık Ltd. Sti, 2002.

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