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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 apopitosis percentage.
Results: The result of the viable cell count in the second and third study groups (98.35±27.3, 97.23±39.38) were low compared to the control group. The results of the proliferative index of study groups (46.47±10.44, 47.23±12.03, 45.55±14.2) were higher than the control group (40.62±9.28). The results of apoptosis in study groups (14.35±1.93, 16.47±7.25, 13.56±5.66) were lower compared to the control group (22.17±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.*
Key words: heparin, colon cancer, DLD-1 cell line.

Some drugs used in cancer treatment lead to tromboembolic 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 neutrophiles, 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 evaluating 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_2 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, tripsinisation procedure was applied to cultured cells to seperate 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_2 containing medium. Five minutes later flasks were removed from the incubater and examined under an inverted microscobe to make sure that adhered cells were seperated. 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 (10^5/200 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_2 containing medium for 72 hours.

Analysis of results

Viable cell ratio: Viable cell ratio in each study group was optained 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 measurment of cellular deoxyribonucleic acid (DNA) content by flow cytometry with the Coulter DNA Prep workstation (Florida USA). Flow cytometry was performed on Coulter Epics Elite flow cytometry (Florida USA). Data were analysed for apoptosis and cell cycle using the Multicycle AV Software (Phoenix Flow System, San Diago, 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

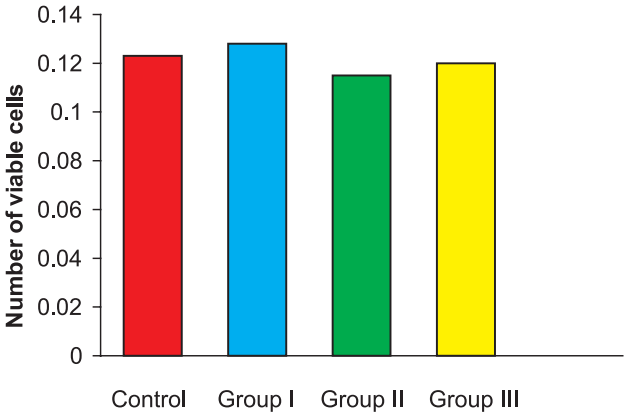

Fig. 1. Comparison of viable cell ratio in each group.

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

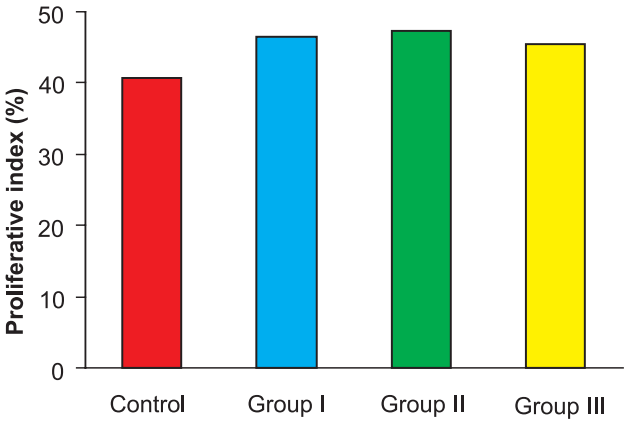

Fig. 2. Comparison of proliferation index (%) in each group.

Proliferation index: The control group and other groups were compared in terms of their proliferation indices. The proliferation index was found 40.62 + 9.28 in the control group, 46.47±10.44 in the group 1, 47.23±12.03 in the group 2 and 45.55±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±15.9 in the control group, 14.35±11.93 in the group 1, 16.47±7.25 in the group 2, 13.56±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).

Tab. 3. Apopitosis (%).

Groups	Apopitosis 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

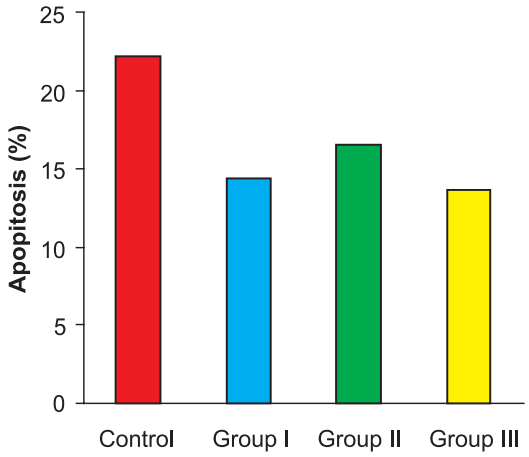

Fig. 3. Comparison of apoptosis percentage in each group.

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 fragmantation (10). Wright et al showed that heparin binds to the 24 kD protease which activates DNA fragmantation in cells going to apoptosis (20). Thus in some experimental models it is thought that inhibition of this enzyme with heparin supresses apoptosis. Maeda et al showed that internucleosomal DNA fragmantation 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 fragmantation in cells. In this study, with the inhibition of the enzyme, which activates DNA fragmentation, it was found that heparin depresses DNA fragmantation 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 disacharides could stimulate or inhibit the cell proliferation in colon cancer cell lines KM12 and KM12SM by affecting the phosphorilation

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 fragmantation 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.

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