

In Vitro Effects of Thermoablation on Apoptosis of Giant cell Tumor of Bone: A Preliminary Report

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Background: Giant cell tumor of bone is a common benign aggressive bone tumor. Recurrent rate in the patients who have the tumor with soft tissue invasion is rather high. Use of strong chemicals such as phenol and liquid nitrogen to lessen the recurrent rate might not be used in this lesion.

Objective: The experiment was carried out to discover apoptotic effects of thermoablation on giant cell tumor of bone to find out a possibility to use thermoablation in the clinic in the patients with extensive lesion.

Material and Method: Tumor cell suspension was prepared from 4 patients who had definite diagnosis of giant cell tumor of bone. The tumor cells were subjected to thermoablation at 45, 47 and 50°C for 10 to 30 minutes before were cultured at 37°C for another 3 days. Osteoblasts and chondrocytes from the last 3 patients were collected, prepared and underwent thermoablation in the same fashion. Apoptosis of tumor cells, chondrocytes and osteoblasts were carried out by the use of flow cytometry.

Results: Thermoablation at 47°C for 30 minutes resulted in < 50% chondrocyte and osteoblast apoptosis and 70-90% tumor cells apoptosis of 3/4 patients. Thermoablation at 47 and 50°C for 20 to 30 minutes has more negative effect on giant cell tumor of bone than chondrocytes and osteoblasts.

Conclusion: Thermoablation might be a useful tool for local tumor control in the clinic.

Keywords: Giant cell tumor; Thermoablation, Apoptosis

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Giant cell tumor of bone is a common benign aggressive bone tumor in Asian patients⁽¹⁻⁴⁾. The tumor can invade and destroy adjacent bone and may result in large bone defect and large soft tissue tumor mass. Recurrent rates after extended intralesional curettage, local treatment with phenol or cryosurgery and reconstruction by the use of allograft or bone cement are still rather high, between 5 and 42 %⁽⁴⁻¹⁰⁾. The patient who has extra osseous extension, Campanacci III, usually has a higher recurrence rate than the ones with only intra osseous lesion, Campanacci I and II. Local treatment by the use of phenol and cryosurgery are the most popular techniques. However, nearby soft tissues including neurovascular bundle, muscle and articular cartilage can be permanently damaged. These

techniques thus might not be suitable for the local control of the tumor with extra-compartment extension and extensive subchondral bone involvement. Placing bone cement before hardening into the tumor lesion after local treatment with phenol can provide local heat to burden the residual tumor cells⁽¹¹⁾. Using bone cement has some limitation as packing bone cement at subchondral bone can result in degeneration of the articular cartilage. Other techniques of local treatment of the tumor are still undergoing investigation, including radiation, mineral trioxide aggregate and bisphosphonates but the numbers of patients with good results are still low⁽¹²⁻¹⁴⁾.

Normal cells produce heat shock proteins (HSPs) which work as a chaperone for proper three dimension folding of the molecule of polypeptides⁽¹⁵⁾. HSPs can stabilize intra cellular polypeptides and prevent the cell from apoptosis under heat stress and other stressful conditions. Under heat stress, normal cells can produce more heat shock proteins via the activation of heat shock factors⁽¹⁵⁾. Thermoablation,

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which is the technique of application of heat stress at a temperature over 40°C to the target cells for a certain period has antiproliferative and apoptogenic associated effects via inducing TNF-related apoptotic ligand (TRAIL)^(16,17). These negative effects were found in some malignancy without compromising immune response⁽¹⁶⁻¹⁹⁾. Thermal effects on cell physiology are strongly dependent on the thermal dose, which are the magnitude of change in temperature and the duration of heat exposure. At higher temperatures, between 45°C and 50°C, a certain amount of osteoblasts still survive and could produce HSPs for self protection⁽²⁰⁾. All normal and tumor cells could be totally destroyed at 55°C to 60°C for the period of 20 to 30 minutes⁽²¹⁻²⁴⁾. This concept incited us to find out the optimum thermal dose for thermoablation in giant cell tumor of bone which could preserve normal chondrocytes and osteoblasts which has not been before reported.

Material and Method

Tumor tissues were collected from 4 patients, one female and three male, who had conventional clinical findings, radiographs and definite diagnosis as giant cell tumor of bone. By the use of standard hematoxylin and eosin pathological study, all patients had confirmed giant cell tumor grade II. The patients' ages ranged between 29 and 52 years. All patients had advance tumor lesion which extended out through the adjacent cortex of the effected bone, Campanacci III. The tumors also broke through the osseous shell of periosteal bone formation at particular areas and extended to the nearby soft tissues in all patients. The tumor was found at distal femur in 1 (male patient number 1), proximal tibia in 1 (female patient number 2), distal ulna in 1 (male patient number 3) and proximal phalanx of the middle finger in 1 patient (male patient number 4). Marginal/wide resection with osseous reconstruction was used in all patients. The articular cartilage in all patients had to be removed with the tumor as the tumor extensively invaded subchondral bone.

To provide consistent tumor cell collection, 2 to 5 cubic centimeters of the solid and avascular part of the tumor mass, which mainly consisted of the tumor cells, were chosen intraoperatively under sterile technique. The tissue was spread on sterile surgical gauzes and was continuously irrigated with normal saline to remove gross contamination of blood and debris. Then, the tissue was immediately transferred in a sterile container containing cell culture media to our cell culture laboratory.

Tumor cell apoptosis after thermoablation at 45, 47 and 50°C for 10, 15, 20 and 30 minutes were investigated by the use of conventional fresh cell culture. One cubic centimeter of the collected tumor tissue was selected and minced into pellets by the use of sterile tissue mincing machine with 0.2 mm diameter blade. Then, the tumor tissue underwent enzymatic digesting by collagenase with a concentration of 0.15 mg/ml in serum free Dulbecco's Modified Eagle Medium (DMEM) cell culture media. The media was supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin. The digestion was carried out at 37°C for 60 minutes. Then, the digested tumor was washed 3 times with 37°C serum free DMEM by centrifugation at 2,000 rpm for 5 minutes to remove most of non tumor cells and debris. The digested tumor tissues were re-suspended in 37°C 10% fetal bovine serum DMEM to extract the tumor cells and resulted in tumor cell suspension. The tumor cell suspension was diluted with 37°C 10% fetal bovine serum DMEM to the concentration of 1×10^6 cells/ml. Haemocytometer was used for tumor cell count. Then the tumor cell suspension was aliquoted for 300 µl, containing 3×10^5 cell/ml, into a 0.5 ml Eppendorf tube. Because of limitation in the amount of the suitable tumor tissue, only thirteen tubes could be prepared from each patient. Each tube was labeled by a serial number for randomization. The tubes with tumor samples were randomly allocated by a computer program, according to their numbers, into 4 groups. One tube in group 1 and four tubes in group 2, four tubes in group 3 and the rest four tubes in group 4.

The tube with tumor suspension in group 1 of all patients was the control. It was incubated in the first carbon dioxide cell culture chamber at 37°C for 30 minutes. From our pilot study, by the use of probe temperature, the temperature of cell culture media rose up very fast and reached the target temperature within one and a half minutes after incubation. Then, the tumor suspension was seeded in a labeled six-well plate. Ten percents fetal bovine serum DMEM with 100 u/ml penicillin and 100 mg/ml streptomycin was added in the well until the total amount of fluid in each well was 2 ml. The plate with tumor suspension was incubated in the second carbon dioxide cell culture chamber at 37°C for 3 days.

In group 2, the tubes containing tumor suspension were randomly allocated into 4 subgroups, subgroup 2.1 to 2.4, with 1 tube in each subgroup. All were incubated in the first cell culture chamber at 45°C, subgroup 2.1 for 10 minutes, subgroup 2.2 for 15

minutes, subgroup 2.3 for 20 minutes and subgroup 2.4 for 30 minutes. In group 3 and 4 the samples were also randomly allocated into 4 subgroups with 1 tube in each subgroup as in the group 2. The tubes with tumor suspension underwent thermoablation as group 2; however, different temperatures were used in each group. In group 3, the incubation temperature was 47°C and in group 4 the incubation temperature was 50°C. The tubes with tumor suspension of the subgroups in group 3 and 4 were also incubated at different times; subgroup 3.1 and 4.1 for 10 minutes, subgroup 3.2 and 4.2 for 15 minutes, subgroup 3.3 and 4.3 for 20 minutes and subgroup 3.4 and 4.4 for 30 minutes, which were the same as the times to group 2.

After thermoablation at different temperatures and times, the tumor suspension in each tube was seeded in a labeled six-well plate. Ten percent fetal bovine serum DMEM with 100 u/ml penicillin and 100 mg/ml streptomycin was added in the well until the total amount of the fluid in each well was 2 ml. All plates were immediately transferred to the second cell culture chamber for further incubation at 37°C for another 3 days, as for the tumor suspension in group 1.

After the 3 days of incubation, apoptosis of tumor cells from all patients was analyzed by measuring cytotoxic activity by the use of cell staining with propidium iodide solution and flow cytometry. The relationship between percentages of tumor cell apoptosis and temperatures/times were analyzed. The supernatant fluids were removed. The tumor cells which were still attached to the well were trypsinized by the use of 0.5% trypsin-EDTA. Then, the trypsinized tumor cell suspension was washed out by the use of 10% DMEM and centrifugation at 2,000 rpm for 5 minutes. The tumor cells were re-suspended in 500 µl of binding buffer in the tube and were stained with 10 µl of propidium iodide solution. The tube was incubated at room temperature for 10 minutes under light protection. Then, cell apoptosis of each tube was determined for fluorescence intensity using a flow cytometer (FACS Calibur, BD Biosciences, USA).

A certain amount of normal cartilage had to be removed with the tumor as the tumor invaded subchondral bone in all patients. Meanwhile a part of normal bone had also been removed with the tumor mass to provided free tumor margins. These cartilage and bone samples were used for the present study of thermoablation representing normal cells. The authors had a limitation in collecting chondrocytes and osteoblasts from the tissue specimens, as the amount of cartilage and bone which had been removed with

the tumor were rather small. Only about one cubic centimeter of each of the tissues was collected from each patient. Thus, only one tube of chondrocyte suspension could be prepared for group 1 and one tube for each subgroup of groups 2, 3 and 4 from a patient. Similar condition had to be applied for osteoblast suspension. The numbers of the tubes containing these normal cell suspension, chondrocyte and osteoblast (from each patient), the allocation of the tubes in each group and subgroup, process of cell extraction and cell suspension, incubating at different temperatures and the times in the first cell culture chamber, re-incubation for 3 days at 37°C in the second cell culture chamber and apoptotic rate measurement, were carried out and analyzed by the same process and method as we have earlier performed for each sample of giant cell tumor.

To determine chondrocytes, anti-collagen type II and anti-aggreant were used. For the identification of osteoblasts, anti-osteocalcin (ab13418, ab13420, ab13421, ab13419), anti-osteopontin ab8448 and anti-collagen type I were used. All data were analyzed by the use of descriptive analysis and Student-t-test, paired and unpaired.

Results

Comparing among tumor cell group 1, osteoblast group 1 and chondrocyte group 1, the lowest percentages of apoptosis were found in the tumor cells, Table 1-3. Percentages of apoptosis of tumor cells of patient number 1, 2 and 3 increased after the thermal dose was increased by raising the temperatures and prolonging the thermoablation times, Table 1 and Fig. 1.

Unfortunately, osteoblasts and chondrocytes from the patient number 1 could not be use in the present study as there was a technical error in the culture of the cells. Only osteoblasts and chondrocytes from the patient number 2, 3 and 4 were used in the present study. Osteoblasts showed similar reaction to the increasing in thermal doses, Table 2 and Fig. 2. However, during thermoablation at the temperature of 47°C and 50°C for 20 and 30 minutes, tumor cells of patient numbers 1, 2 and 3 showed significant higher percentages of apoptosis than those of the osteoblast group, Fig. 1 and Fig. 2. The largest differences in terms of percentage of cell apoptosis between tumor cells and osteoblasts were observed during thermoablation at 47°C for 20 and 30 minutes. On the other hand, percentage of apoptosis was low in chondrocyte group 1 of all patients and the apoptosis did not significantly

Table 1. Percentages of giant tumor cell apoptosis by the use of propidium iodide (PI) at various temperatures and times

Conditions/Groups Cells from patient No.	37°C, Gr. 1			45°C, Gr. 2			47°C, Gr. 3			50°C, Gr. 4		
	10 min	15 min	20 min	30 min	10 min	15 min	20 min	30 min	10 min	15 min	20 min	30 min
1	2.1	2.2	2.3	2.4	3.1	3.2	3.3	3.4	4.1	4.2	4.3	4.4
2	16.8	17.9	25.7	37.6	72.3	68.4	72.4	76.4	88.3	83.1	85.5	83.5
3	5.3	3.7	5.3	7.6	24.4	44.6	58.5	80.1	83.9	81.8	79.2	80.5
4	20.8	22.6	28.2	45.2	67.1	74.3	78.6	81.5	82.9	79.6	79.9	83.9
Mean	14.3	14.7	19.7	30.1	54.6	62.5	69.8	79.5	85.0	81.5	81.5	82.6
SD	6.6	8.0	12.6	16.2	21.4	12.8	10.3	2.2	2.3	1.4	3.4	1.5
4	7.7	10.5	15.5	18.4	31.6	42.7	44.4	61.0	65.6	61.7	53.8	49.5

Table 2. Percentages of osteoblast apoptosis by the use of propidium iodide (PI) at various temperatures and times

Conditions/Groups Patient number	37°C, Gr. 1			45°C, Gr. 2			47°C, Gr. 3			50°C, Gr. 4		
	10 min	15 min	20 min	30 min	10 min	15 min	20 min	30 min	10 min	15 min	20 min	30 min
2	2.1	2.2	2.3	2.4	3.1	3.2	3.3	3.4	4.2	4.2	4.3	4.4
3	26.0	24.7	28.3	33.6	41.9	40.7	50.1	60.4	66.7	74.7	80.7	88.7
4	26.7	31.7	34.2	33.7	44.2	35.5	46.9	50.6	58.1	76.3	84.8	89.5
Mean	27.4	33.6	31.8	26.6	51.3	58.1	49.5	50.9	66.1	75.5	75.0	91.0
SD	26.7	30.0	31.4	31.3	45.8	44.8	48.8	53.9	63.6	75.5	80.2	89.8
4	0.5	3.8	2.9	3.3	3.9	9.6	1.7	4.6	3.9	0.6	4.9	0.9

Table 3. Percentages of chondrocyte apoptosis by the use of propidium iodide (PI) at various temperatures and times

Conditions/Groups Patient number	37°C, Gr. 1			45°C, Gr. 2			47°C, Gr. 3			50°C, Gr. 4		
	10 min	15 min	20 min	30 min	10 min	15 min	20 min	30 min	10 min	15 min	20 min	30 min
2	2.1	2.2	2.3	2.4	3.1	3.2	3.3	3.4	4.2	4.2	4.3	4.4
3	9.8	22.6	23.4	17.4	15.2	15.0	15.8	27.8	21.3	15.7	23.9	22.9
4	24.7	22.1	23.7	28.3	19.8	15.3	17.9	27.5	18.3	18.2	14.8	21.4
Mean	18.7	21.1	20.6	15.9	13.0	15.2	15.9	15.0	25.9	37.3	22.6	19.1
SD	17.7	21.9	22.6	20.5	15.9	15.2	16.5	23.5	21.8	23.7	20.5	21.2
4	6.2	0.6	1.7	5.5	2.8	0.1	1.2	5.9	3.1	9.6	4.9	1.6

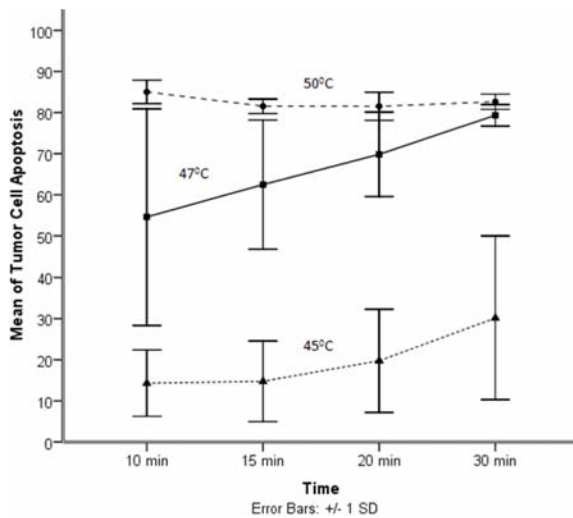


Fig. 1 Relationship between mean apoptosis and thermoablation times of giant cell tumor of the patient number 1, 2 and 3, the good thermoablation responder, at 45, 47 and 50°C

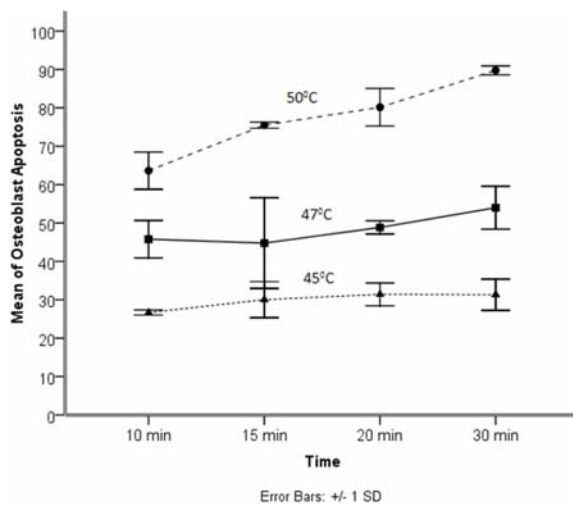


Fig. 2 Relationship between mean apoptosis and thermoablation times of osteoblasts at 45, 47 and 50°C

change after thermal dose was increased, Table 3 and Fig. 3.

Tumor cells of the patient number 4 responded poorly to thermoablation, Table 1 and Fig. 4. About 50% of the tumor cells of patient number 4 still survived at the temperatures of 47°C and 50°C for 20 and 30 minutes.

Discussion

Only tumor cells of patients who had giant

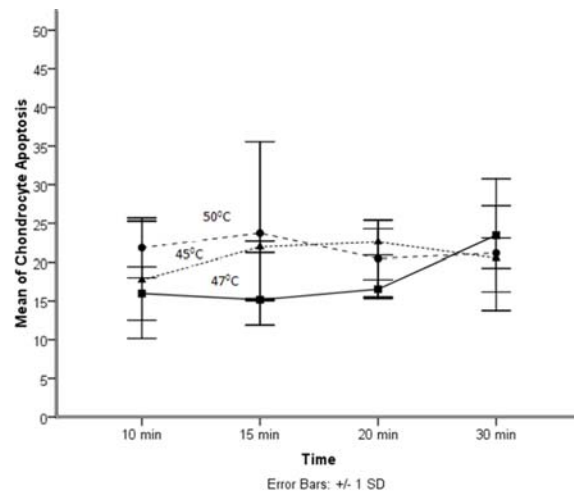


Fig. 3 Relationship between mean apoptosis and thermoablation times of chondrocytes at 45, 47 and 50°C

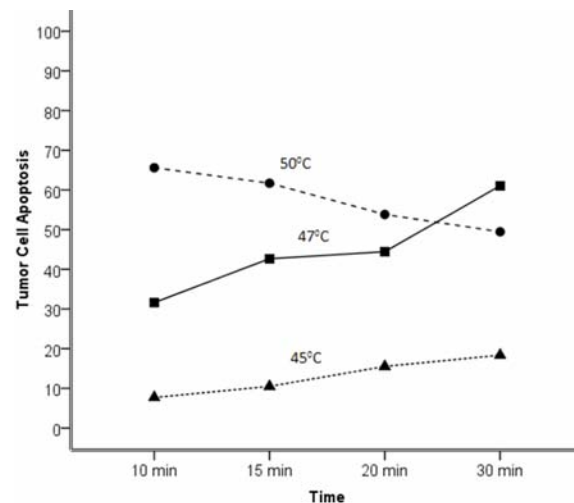


Fig. 4 Relationship between mean apoptosis and thermoablation times of giant cell tumor of the patient number 4, the poor responder at 45, 47 and 50°C

cell tumor of long bones, Campanacci grade III, were enrolled in the present study because these patients had aggressive lesion for which conventional intralesional extended curettage and the use of phenol as a local treatment might not be suitable. Local treatment with phenol could leak out through the bony defect and destroy surrounding vital soft tissues, including nearby neurovascular bundles. Thus, intralesional extended curettage with local thermoablation was planned to be used in the patients in the future. In order to use the technique in the clinic, optimum thermal

dose in terms of temperature and timing have to be investigated *in vitro*. The numbers of sample in our study was rather limited because of technical error so all data were present in detail and the sample from each patient were presented separately.

As all of the presented patients had giant cell tumor grade II by histopathological classification, this similarity could reduce the variation of cell types and might also minimize the deviation in the measurement of apoptosis after thermoablation. Only the solid and avascular part of the tumor tissue was carefully chosen for the study to minimize the number of non tumor cell. Although tumor suspension in our study consisted of tumor mononuclear cells, giant cells and non tumor cells, the numbers of non tumor cells should be small because of multiple steps of washing and centrifugation which also could remove most of non tumor cells from the tumor cell suspension. The cell line of the tumor was not used because they have passed through many subcultures and might have changed their biological response to thermoablation. The crude cell samples were used to make the present study as close to the real conditions as possible.

The amount of solid and avascular tumor tissue, cartilage and bone which have been collected during the surgery were rather limited. So, only thirteen tubes of cell suspension could be prepared from each patient and only one tube was available for group 1 and each subgroup in the group 2, 3 and 4. This condition was the weak point of the present study. However, the tubes were allocated into the designed groups randomly to minimize selection bias.

Bone cement can produce a certain amount of thermal dose during polymerization and hardening. The surface temperature of the hardening bone cement temperature may rise up to above 60°C^(11,23-25). Komiya et al, reported degeneration of a cell line of mononuclear cells from giant cell tumor of bone after an exposure to hyperthermic condition at the temperature of 60°C for 10 minutes⁽¹¹⁾. However, the period of heat production by polymerization of bone cement, including peak temperature and temperatures above 50°C, is usually less than 5 minutes⁽²⁵⁾. This limited time of thermoablation could limit the amount of thermal dose which might not be able to destroy the tumor cells in the deep layers which were our target. Furthermore, temperatures higher than 50°C could produce significant negative effects on normal bone and cartilage cells. Therefore, the authors studies lower temperature thermoablation to lessen the negative effects on normal cells but used longer periods of thermoablation, between 20 and 30

minutes, to destroy the residual tumor cells in both superficial and deep layers after extended intralesional curettage.

Tumor cells could maintain their growth and survival better than normal cells as lowest percentage of cell apoptosis was found in tumor cell group 1, comparing to chondrocyte and osteoblast group 1, Table 1-3. However, after thermoablation between 47°C and 50°C with the periods of 20 and 30 minutes, chondrocytes and osteoblasts had lower apoptosis than giant cell tumor, Table 1, 2 and 3. About 70 to 90% apoptosis were found in the tumor suspension of the patient numbers 1, 2 and 3, Fig. 1; meanwhile more than 50% of chondrocytes and osteoblasts still survived, Fig. 2 and 3. Percentages of tumor cell apoptosis directly related to the thermal doses. The higher the temperature and the longer the thermoablation time, the higher the percentages of apoptosis that were found in tumor cells and osteoblasts, Fig. 1 and 2. These results gave us a new technique of local treatment of the giant cell tumor of bone which might have fewer negative effects on the surrounding normal tissues. Use of heat at a temperature of 47°C for 20 to 30 minutes should be the most suitable thermoablation for giant cell tumor of bone. However, thermoablation could not induce apoptosis in all giant cell tumor samples. The tumor cell suspension of patient number 4 responded poorly to thermoablation, Fig. 4. A preoperative thermoablation test *in vitro* should be performed before definitive extended intralesional curettage and thermoablation is planned. Only thermosensitive tumor should be treated locally by thermoablation.

Interestingly, chondrocytes could withstand thermoablation much better than the tumor cells and osteoblasts. Thermoablation might be a good tool for the management of giant cell tumor which has subchondral bone invasion, as the chondrocytes could be preserved better than through the use of phenol or cryotherapy.

Conclusion

Thermoablation at the temperature of 47°C for 30 minutes significantly induce apoptosis in giant cell tumor of bone in a certain number of samples, with less negative effect on chondrocytes and osteoblasts. Thermoablation may be a useful tool as an adjuvant local treatment in giant cell tumor of bone.

Potential conflicts of interest

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รายงานผลการทดลองอิทธิพลของความร้อนต่ออัตราการตายของเซลล์เนื้ออกของกระดุกชนิด ไจแอนท์เซลล์

ระพีพัทธ์ พิมลสานต์, กฤษณ์ เจริญลาภ, บรรจบ อริยะบุญศิริ, อติศักดิ์ ว่องขจรศิลป์, สารเนตร์ ไวกกุล

ภูมิหลัง: เนื้ออกกระดุกชนิดไจแอนท์เซลล์เป็นเนื้ออกที่ร้ายที่พบได้บ่อยและมักทำลายกระดุกเป็นบริเวณกว้าง ทำให้กระดุกหักและอาจลุกลามสู่เนื้อเยื่ออ่อนโดยรอบ ในผู้ป่วยที่มีรอยโรคที่ลุกลามมักเกิดมีเนื้ออกนี้ขึ้นอีก หลังการรักษา การใช้ฟินอร์เพื่อลดการเกิดขึ้นใหม่ในรายเช่นนี้อาจก่อให้เกิดการทำลายเซลล์กระดุก เซลล์กระดุกอ่อน หลอดเลือดและเส้นประสาทข้างเคียง

วัตถุประสงค์: ศึกษาหาความเป็นไปได้ของการใช้ความร้อนที่ไม่สูงนักระหว่าง 45 ถึง 50 องศาเซลเซียสเพื่อใช้ทำลายเนื้ออกที่ลุกลามสู่เนื้อเยื่ออ่อนโดยไม่ทำลายเซลล์กระดุกและเซลล์กระดุกอ่อน ก่อนนำวิธีการนี้มาใช้ทางคลินิก

วัสดุและวิธีการ: นำเซลล์เนื้ออกไจแอนท์เซลล์จากผู้ป่วย 4 ราย มาเตรียมให้เป็นเซลล์แขวนลอยในน้ำเลี้ยงเซลล์ แล้วให้ความร้อนกับเซลล์เนื้ออกที่อุณหภูมิ 45, 47 และ 50 องศาเซลเซียส เป็นเวลาระหว่าง 10 และ 30 นาที แล้วเลี้ยงเซลล์ต่ออีก 72 ชั่วโมง วัดการตายของเซลล์โดยวิธีไพร์โซโตเมตตรี และการย้อมด้วย โปลิเดียม เปรียบเทียบกับเซลล์กระดุกและเซลล์กระดุกอ่อนซึ่งได้รับการเตรียมอย่างเดียวกัน

ผลการศึกษา: ผลการศึกษาพบว่าที่อุณหภูมิ 47 องศาเซลเซียสและเวลา 30 นาที เซลล์เนื้ออกจากผู้ป่วย 3 ใน 4 ราย ตายกว่าร้อยละ 70 ถึง 90 ในขณะที่เซลล์กระดุกและเซลล์กระดุกอ่อนตายน้อยกว่าร้อยละ 50

สรุป: อาจนำวิธีการใช้ความร้อนนี้มาใช้ทำลายเนื้ออกไจแอนท์เซลล์ที่ลุกลามมาก เพื่อลดการทำลายเซลล์ปกติ
