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The effect of temperature and duration of pasteurization exposure on tumor viability in osteosarcoma

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***Abstract*---**This study aims to examine the effect of temperature and duration of pasteurization exposure on osteosarcoma tumor tissue degeneration, necrosis, and tumor cell viability. Using osteosarcoma tumor tissue, researchers evaluated degeneration, necrosis, and tumor cell viability following exposure to pasteurization. Hematoxylin-eosin (HE) staining was utilized to assess degeneration and necrosis, whereas CKAP4 immunohistochemical (IHC) staining was utilized to assess cell viability. The Kruskal-Wallis test showed a significant increase in degenerated tumor cells ($p = 0.004$) with the highest number found in the 60°C temperature group with a 40-minute exposure time, and a significant increase in necrotic tumor cells ($p = 0.000$) with the highest number found in the 70°C temperature group with a 40-minute exposure time. The 70°C for 40 minutes group had the fewest viable tumor cells ($p = 0.000$). This study found that the dose of pasteurization exposure had an effect on osteosarcoma cell degeneration, necrosis, and viability. With increasing temperature and duration of pasteurization, tumor cells exhibited increased degeneration and necrosis, as well as decreased viability, with the

optimal exposure occurring at 70°C for 40 minutes, which could kill all tumor cells.

***Keywords*---**osteosarcoma, degeneration, necrosis, viability, pasteurization.

Introduction

Pasteurization is a bone recycling technique by reusing the resected bone after being heated in a saline solution (Zekry et al., 2019). It is known that high temperatures can disrupt cell membranes, protein denaturation, and swelling of organelles which can eventually lead to cell death (Török et al., 2014). Tumor cells have a higher sensitivity to temperature changes because they capture more heat than normal cells. This is the principle of pasteurization, where heating in a specific temperature range can cause tumor cell death without affecting the normal surrounding tissue function physiologically (Lin & Baehrecke, 2015). After exposure to pasteurization, tumor cells can undergo degeneration, apoptosis, and necrosis, but viable tumor cells can still be obtained. Tumor cells that undergo degeneration and necrosis due to cell damage after exposure to pasteurization can be assessed histopathologically (Chui et al., 2016).

There is still disagreement on the optimal temperature and duration of pasteurization as the standard of therapy. Research conducted by Rong & Mack (2000) on osteosarcoma cells in vitro stated that exposure to a temperature of 43.5°C could induce apoptosis, and higher temperature increased tumor cell death through necrosis mechanism. However, according to a study conducted by Sakayama et al. (2004), no tumor cells were found in post-pasteurized specimens exposed to a temperature of 60°C for 30 minutes. This finding is supported by the cytocidal effect of heating which can only be achieved at a minimum temperature of 60°C. In another study, Watanabe used pasteurization exposure at 70°C to ascertain the lethal effect on tumor cells, but at this temperature cells began to show signs of damage and denaturation of BMP (Watanabe et al., 2003). This finding is also supported by result of a study by Yasin et al where at temperature of 80° to 134°C, the ability of graft to regenerate would decrease. Another study found that exposure to 70°C for more than 1 hour can damage BMP (Hakki et al., 2014; Urist & Iwata, 1973). At 60-65°C, heating for 40 minutes gave satisfactory results with the failure of tumor tissue to grow in culture (Kode et al., 2014), while Rong et al. (1995) stated that at 60°C for 20 and 30 minutes, it was enough to kill all the tumor cells.

Based on previous research, it is estimated that the optimal temperature and duration for pasteurization is in the temperature range of 60°C-70°C, with a duration of 20-40 minutes. However, there is no literature and research on the optimal duration and temperature of pasteurization needed to kill all tumor cells while maintaining osteoinductive ability. Therefore, this study aims to analyze the effect of temperature and duration of pasteurization exposure on tumor cell degeneration, necrosis, and viability in osteosarcoma tumor tissue.

Materials and Methods

This study used an experimental laboratory design to evaluate post-pasteurized grafts tumor viability. Osteosarcoma tissue which is derived from patients who have not been treated with chemotherapy were used as a sample (Kuijjer et al., 2013). Approximately 2,5 cm^3 of tissue within the active zone of osteosarcoma amputate were used as a sample. These tissues are then pasteurized on different degree and duration. There are two types of temperature (60°C and 70°C) and three types of duration (20, 30, and 40 minutes). Resulting in six groups. Additionally, untreated sample were used as negative control. After pasteurization, the tissues are then stained using hematoxylin-eosin to evaluate the tumor degeneration and necrosis, while tumor viability is evaluated using immunohistochemical staining. Histopathological staining is done using the procedure described below:

Tissue Processing Stage

There are several processes: dehydration, clearing, and impregnation stages using a tissue processor and embedding. The stages of dehydration were carried out by gradually adding the specimen in alcohol, starting from 70% to 96% alcohol. The clearing step was carried out by inserting the specimen in xylol. The impregnation step was carried out by placing the specimen in solid paraffin at 60°C for 2 hours. Finally, the embedding stage is carried out by preparing the base mold and cassette at a temperature of 60°C, then placing it on a cold plate for up to 2-4 minutes.

Hematoxylin-eosin (HE) staining

The paraffin blocks were mounted on a microtome and cut into thin slices. The slices were attached to a microscope slide then the first staining step was started by de-waxing using a solvent to remove wax from the slides prior to staining. HE staining uses two dyes, hematoxylin, and eosin. After the coloring is complete, then it is covered with cover glass.

Immunohistochemical (IHC) staining

Monoclonal antibodies were used against rough endoplasmic reticulum (rER) cytoskeleton-linking membrane protein P63 (CLIMP-63). The cytoplasm contains many rough endoplasmic reticulum (rER) in viable cells, which will bind to IHC dye and give color to the cytoplasm. In contrast, necrotic cells will have a clear cytoplasm. Next, the counting process is carried out with a light microscope to count live cells (stained).

Statistical analysis

The collected cell viability, necrosis, and degeneration data will be described descriptively by looking at the mean and standard deviation in the 60°C and 70°C temperature groups and the exposure with a duration of 20, 30, and 40 minutes. In addition, statistical analysis was also conducted. Normality test is conducted using Saphiro Wilk test. If the result is normal then we will proceed to analyze

each treatment groups effect significancy using ANOVA, otherwise we would use Kruskal-Wallis test. If significant difference using Kruskal-Wallis is found, post hoc test is then conducted afterwards to pinpoint each different treatment group's effect on each parametric. For all statistical analysis, value of <0.05 is accepted as significant. This statistical analysis is done using the SPSS 26 program.

Results and Discussions

Histopathological reading of tissues on different groups of treatment were analyzed after stained. Sample of histopathological reading is shown at Figure 1.

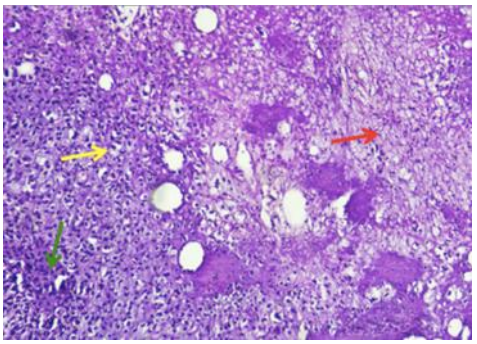

Figure 1. Histopathological reading result after HE staining. From the slide we can observe: (A) necrotic cells (red arrow), (B) degenerated cells (yellow arrow), (C) viable cells (green arrow)

Tumor Cell Degeneration

Tumor degeneration result on different treatment group is described on Table 1. From the table it is evident that more degeneration can be observed in samples treated with longer duration of pasteurization. Statistical analysis using Saphiro Wilk determined that the data is abnormally distributed ($p<0.05$) and therefore Kruskal Wallis test is conducted. This test confirmed the effect of pasteurization by showing significant difference between tumor cell degeneration on different treatment group ($p=0.004$). Accordingly, post hoc analysis is then conducted and is tabulated on Table 2.

Post hoc analysis show that all treatment group (60°C and 70°C) resulted in significant difference compare to control group. Comparing between treatment groups, significant difference can be found on 40 minutes of 70°C compared to shorter duration of pasteurization at 70°C. This study show that the tumor degeneration in 60°C treatment group were higher than in 70°C treatment group. Protein degeneration happens on environment with temperature 60°C, while higher temperature increased tumor cell death through necrosis mechanism (Zhang et al., 2018). This condition explained that higher temperature exposure will lead to more necrotic cell than degenerated cell (Rong & Mack, 2000).

Table 1
Tumor cell degeneration on different treatment group and Kruskal Wallis Test Analysis result

	Temperature (°C)	Duration (minutes)	n	Mean	SD	P value
Tumor Cell Degeneration	60°C	20	8	19.37	11.47	
		30	8	23.12	21.86	
		40	8	27.50	24.92	
	70°C	20	8	12.50	9.25	0.004
		30	8	13.12	5.31	
		40	8	26.25	6.94	
		Control	8	1.88	3.72	

Table 2
Post Hoc analysis comparing each different treatment group effect on the degeneration of tumor cells

Temperature (°C)		60°C			70°C			
	Duration (minutes)	20	30	40	20	30	40	Control
60°C	20		0.676[a]	0.422[a]	0.208[a]	0.184[a]	0.169[a]	0.005[b*]
	30	0.676[a]		0.715[a]	0.236[a]	0.245[a]	0.710[a]	0.028[b*]
	40	0.422[a]	0.715[a]		0.145[a]	0.151[a]	0.895[a]	0.021[b*]
70°C	20	0.208[a]	0.236[a]	0.145[a]		0.871[a]	0.005[a*]	0.010[b*]
	30	0.184[a]	0.245[a]	0.151[a]	0.871[a]		0.001[a*]	0.001[b*]
	40	0.169[a]	0.710[a]	0.895[a]	0.005[a*]	0.001[a*]		0.000[b*]
	Control	0.005[b*]	0.028[b*]	0.021[b*]	0.010[b*]	0.001[b*]	0.000[b*]	

[a]Independent sample t-test [b]Mann-Whitney *significant ($p<0.05$)

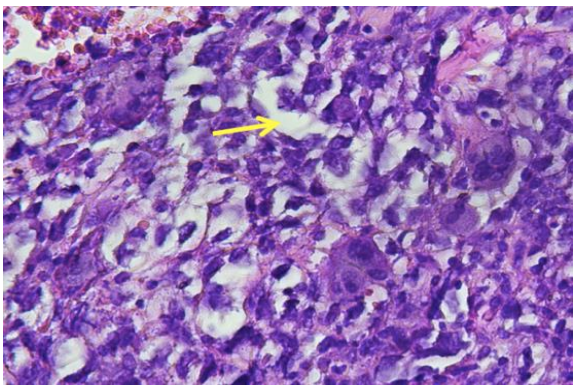

Figure 2. Histopathological result after HE staining. From the slide we can observe ballooning in degenerated cells (yellow arrow)

Tumor Cell Necrosis

Tumor necrosis result on different treatment group is described on Table 3. Similar to cell degeneration, from the table it is evident that more necrosis can be

observed in samples treated with longer duration and higher temperature of pasteurization. Statistical analysis using Saphiro Wilk determined that the data is abnormally distributed ($p<0.05$) and therefore Kruskal Wallis test is performed. This test confirmed the effect of pasteurization by showing significant difference between tumor cell necrosis on different treatment group ($p=0.000$). Accordingly, post hoc analysis is then conducted and is tabulated on Table 4. Similar to tumor cell degeneration, post hoc analysis show that all treatment group (60°C and 70°C) resulted in significant difference in comparison to control. Comparing between treatment groups, significant difference can be observed on almost all other treatment groups with most necrotic cell tumor can be found on 40 minutes of 70°C treatment group.

Table 3
Tumor cell necrosis on different treatment group and Kruskal Wallis Test Analysis result

	Temperature (°C)	Duration (minutes)	n	Mean	SD	P value
Tumor Cell Necrosis	60°C	20	8	40.93	16.14	0.000
		30	8	50.00	22.83	
		40	8	63.62	29.96	
	70°C	20	8	51.25	24.31	
		30	8	58.75	23.41	
		40	8	73.12	8.42	
		Control	8	5.63	8.21	

Table 4
Post Hoc analysis comparing each different treatment group effect on the necrosis of tumor cells

Temperature (°C)		60°C			70°C			
	Duration (minutes)	20	30	40	20	30	40	Control
60°C	20		0.375[a]	0.087[a]	0.335[a]	0.098[a*]	0.003[b*]	0.001[b*]
	30	0.375[a]		0.324[a]	0.917[a]	0.462[a]	0.023[b*]	0.001[b*]
	40	0.087[a]	0.324[a]		0.380[a]	0.722[a]	0.460[b]	0.001[b*]
70°C	20	0.335[a]	0.917[a]	0.380[a]		0.540[a]	0.026[b*]	0.001[b*]
	30	0.098[a]	0.462[a]	0.722[a]	0.540[a]		0.236[b]	0.001[b*]
	40	0.003[b*]	0.023[b*]	0.460[b]	0.026[b*]	0.236[b]		0.001[b*]
	Control	0.001[b*]	0.001[b*]	0.001[b*]	0.002[b*]	0.001[b*]	0.001[b*]	

[a]Independent sample t-test [b]Mann-Whitney *Significant ($p<0.05$)

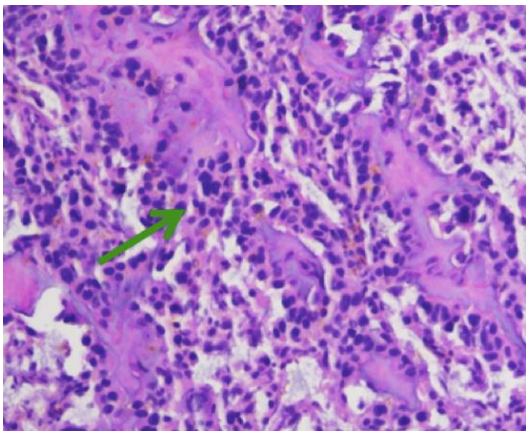

Figure 3. Histopathological result after HE staining. From the slide we can observe viable cells (green arrow)

Tumor Cell Viability

Tumor viability result on different treatment group is described on Table 5. From the table it can be observed that there is a huge difference on tumor viability treated with longer duration and higher temperature of pasteurization. The result show there is no viable cell observed on tissues treated with pasteurization for 40 minutes at 70oC. Statistical analysis using Saphiro Wilk determined that the data is abnormally distributed ($p<0.05$) and therefore Kruskal Wallis test is conducted. This test confirmed the effect of pasteurization by showing significant difference between viable tumor cell on different treatment group ($p=0.000$). Accordingly, post hoc analysis is then conducted and is tabulated on Table 4. Post hoc analysis show that all treatment group (60°C and 70°C) resulted in significant difference in comparison to control. Comparing between treatment groups, significant difference can be observed on almost all other treatment groups compared to 40 minutes of 70°C treatment group.

Table 5
Tumor cell viability on different treatment group and Kruskal Wallis Test Analysis result

	Temperature	Duration	n	Mean	SD	P value
Tumor Cell Viability	Temperature 60^{o}C	20 Minute	8	26.88	15.79	0.000
		30 Minute	8	12.5	23.76	
		40 Minute	8	5	4.63	
	Temperature 70^{o}C	20 Minute	8	18.75	28.63	
		30 Minute	8	3.75	10.61	
		40 Minute	8	0	0	
		Control	8	92.5	11.65	

Table 6
Post Hoc analysis comparing each different treatment group effect on the viability of tumor cells

Temperature (°C)		60°C			70°C			
	Duration (minutes)	20	30	40	20	30	40	Control
60°C	20		0.034[a*]	0.003[a*]	0.151[a]	0.002[a*]	0.000[a*]	0.001[a*]
	30	0.034[a*]		0.865[a]	0.822[a]	0.158[a]	0.027[a*]	0.001[a*]
	40	0.003[a*]	0.865[a]		0.825[a]	0.102[a]	0.010[a*]	0.001[a*]
70°C	20	0.151[a]	0.822[a]	0.825[a]		0.125[a]	0.027[a*]	0.001[a*]
	30	0.002[a*]	0.158[a]	0.102[a]	0.125[a]		0.317[a]	0.000[a*]
	40	0.000[a*]	0.027[a*]	0.010[a*]	0.027[a*]	0.317[a]		0.000[a*]
	Control	0.001[a*]	0.001[a*]	0.001[a*]	0.001[a*]	0.000[a*]	0.000[a*]	

[a]Mann-Whitney *Significant ($p<0.05$)

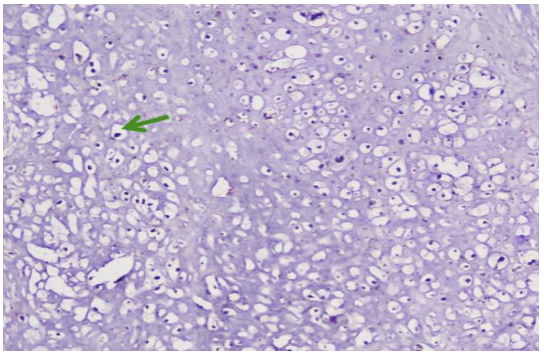

Figure 4. Histopathological result after IHC staining. From the slide we can observe non viable cell with clear cytoplasm (green arrow)

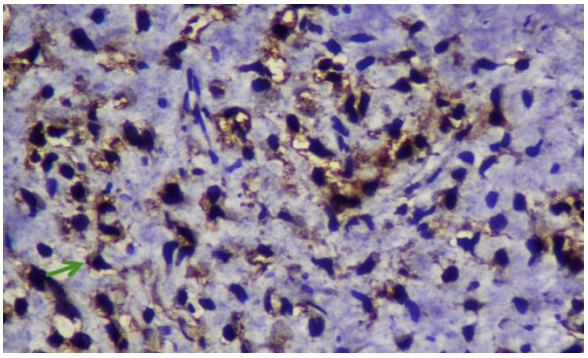

Figure 5. Histopathological result after IHC staining. From the slide we can observe viable cell with moderate-strong intensity (green arrow)

It is not infrequently that non viable tumor cell with intact cell membrane is difficult to distinguish with the viable ones in HE staining. In this condition, examination using IHC staining has a high accuracy level to evaluate viability of tumor cells compared to HE staining. IHC staining on this research used antibody toward cytoskeleton-linking membrane protein P63 (CLIMP-63). CLIMP-63 is a

rough endoplasmic reticulum (rER) which has a function for transporting protein. Abundant ribosomes and rough endoplasmic reticulum (rER) can be observed within viable tumor cell. The antigen-antibody binding between CLIMP-63 and monoclonal antibody of CKAP4 will color the cytoplasm, on the other hand necrotic cells will have clear cytoplasmic. These eases distinguishing necrotic and viable tumor cells (Hookway et al., 2017).

There are few mechanisms that explains the correlation between temperature and tumor cells necrosis. Among them are the fact that if tissue's temperature is raised way above its physiological temperature, it will result in the change of protein structure and organelle. Cytoskeletal component of the cells will be deranged and result in changes of cell membrane permeability increasing the intracellular Na+, H+, dan Ca2+. This consequently result in the delay of DNA synthesis and transcription as well as RNA translation. Cell cycle will stop because denaturation of protein. Finally, the cell will degrade through proteasomal and lisosomal pathway (Hildebrandt et al., 2002; Orgill et al., 2006).

As previously mentioned that protein degeneration happens on environment with temperature 60°C, consequent increase in cellular temperature will increase cell necrosis exponentially. Temperature level necessary to induce cell death varies and depends on the time of exposure and the amount of temperature. Higher temperature increased tumor cell death through necrosis mechanism (Zhang et al., 2018). In this study, cell degeneration result was higher in 60°C treatment group, while cell necrosis result was higher in 70°C treatment group. This result is in accordance with previous studies where at a lower temperature of 60°C tumor cells will begin to degenerate and increasing temperature to 70°C led to more cells necrosis because there has been more severe disruption and cell damage due to thermal exposure (Watanabe et al., 2003). In his research, Watanabe explains that specimens which are pasteurized at 60°C could kill tumor cells. Despite so, some viable tumor cells can still be found. Therefore, Watanabe et al. (2003) also tried increasing the pasteurization temperature to 70°C to ensure the lethal effect to tumor cells is achieved (Teixeira et al., 2019).

In this study, exposure of pasteurization for 40 minutes at 70oC increase the tumor cell degeneration and necrosis as well as decrease tumor viability. This dose also can achieve tumor eradication which is important in limb salvage surgery using sterillized tumor bone as autograft to fill the defect after wide resection. As the exposure to temperature of 70^{o}C did not affect the biomechanic strength and osteoconductivity (Manabe, 1993), we suggest using exposure of pasteurization for 40 minutes at 70^{o}C as optimal dose to sterilize the tumor bone for reconstructive technique using recycle bone in limb salvage surgery.

Conclusion

Increased tumor cell degeneration and necrosis, as well as decreased tumor cell viability was found with increasing temperature and duration of pasteurization. In comparison to other treatment groups, significant difference can be observed mainly on those pasteurized using 70oC for 40 minutes compared to other treatment groups. The exposure at 70°C for 40 minutes is the optimal dose to

achieve tumor eradication with no viable tumor cells whilst preserving innate osteoinduction capability.

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Conflict of Interest

The authors declare no conflict of interest

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