CORRELATION BETWEEN THE DURATION OF LIQUID NITROGEN EXPOSURE WITH THE VIABILITY OF TUMOR TISSUE IN OSTEOSARCOMA PATIENTS AT DR SOETOMO GENERAL HOSPITAL PERIOD 2020

Haris Dwi Khoirur Rofiq1, Ferdiansyah2, Sjahjenny Mustokoweni3
1,2Department of Orthopaedic and Traumatology, Faculty of Medicine Universitas Airlangga-Dr. Soetomo General Hospital, Surabaya
3Department of Pathology Anatomy, Faculty of Medicine Universitas Airlangga-Dr. Soetomo General Hospital, Surabaya

ABSTRACT
Osteosarcoma is the most common primary malignant bone tumor, but its incidence is very rare, about 1 in 200,000 compared to other malignant neoplasms such as breast cancer and lung cancer. The use of liquid nitrogen graft has advantages compared to other methods, namely bone segments that are given liquid nitrogen have better osteoinductive and osteoconductive abilities, the size and shape of the bones are more compatible for reconstruction, and the procedure is easier than other methods. In a study conducted by Rahman et al (2009) and Igarashi et al (2014) the bone immersion process using liquid nitrogen was carried out for 20 minutes so as to obtain both clinical and histological results. There were 5 treatment groups in osteosarcoma tissue that were treated with cryosurgery, namely the osteosarcoma group with cryosurgery treatment 10, 15, 20, 25, and 30 minutes. At the 10th and 15th minute groups still obtained tumor viability with results of 30.5% and 12.25% respectively on staining. Hematoxylin Eosin and 24.9% and 9.6% on immunohistochemical examination. Tumor viability was not obtained or 0% in the 20, 25 and 30 min groups on Hematoxylin Eosin staining and on staining immunohistochemistry. The best minimum time for cryosurgery for primary bone tumor cell therapy is 20 minutes.

Keywords : Liquid Nitrogen, Viability of Tumor Tissue, Osteosarcoma

I. INTRODUCTION
Osteosarcoma is the most common primary malignant bone tumor, but its incidence is very rare, about 1 in 200,000 compared to other malignant neoplasms such as breast cancer and lung cancer. Reconstruction using biological materials has been developed such as allogeneic bone grafts, vascular and nonvascular autografts, autologous bone extensions, and sterilized bone grafts. However, the sterilization process of the graft using autoclave, irradiation and pasteurization can affect the quality of the bone graft(Yamamoto & Tsuchiya, 2017).

The use of liquid nitrogen grafts has advantages compared to other methods, namely bone segments that are given liquid nitrogen have better osteoinductive and osteoconductive abilities, the size and shape of the bones are more compatible for reconstruction, and the procedure is easier compared to other methods(Tanzawa et al., 2009). A temperature of at least -20°C has resulted in a significant increase in the local tumor recurrence rate and a sharp decrease in the incidence of associated complications(Ralph C Marcove, Weis, Vaghaiwalla, Pearson, & Huvos, 1978). In a study conducted by Igarashi et al also obtained satisfactory results in patients with malignant bone tumors who underwent reconstruction of bone defects using autographs frozen with liquid nitrogen at the Enneking score of more than 90% of patients in this study and only 8% of patients experienced recurrence local tumor(Igarashi et al., 2014). In a study conducted by Rahman et al (2009) and Igarashi et al (2014) the bone immersion process using liquid nitrogen was carried out for 20 minutes so as to obtain both clinical and histological results.
This study aims to determine the differences in morphology and viability of tumor tissue histopathologically using Hematoxylin Eosin and immunohistochemically using and CKAP4 Osteosarcoma patients at Dr Soetomo Hospital in 2020 after exposure to liquid nitrogen within 10 minutes, 15 minutes, 20 minutes, 25 minutes, and 30 minutes.

II. METHODS

This study is an experimental laboratory study and the sample was an osteosarcoma tissue that has been given exposure to liquid nitrogen for a certain duration at Dr. Soetomogeneral hospital period 2020. A piece of tissue was taken from the amputate which was then given exposure to liquid nitrogen with different durations. The tissue pieces are then sent to the anatomical pathology laboratory and tissue bank installation for viability tests.

Processing tissue or specimens with the paraffin method through the stages of dehydration, clearing, and impregnation using a tissue processor and embedding. Dehydration is done by adding the specimen in alcohol gradually starting from 70% to 96% alcohol. The clearance 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.

The embedding stage is carried out by preparing the base mold and cassette at a temperature of 60°C. Next, the paraffin dispenser faucet is pressed on the base mold until the volume is sufficient. The specimen is then inserted into the base of the mold using tweezers. The cassette is placed on a base mold that has been filled with tissue/specimen. The filled base mold is placed on a cold plate for up to 2-4 minutes and the base mold makes a thik sound. After that, the cassette is removed from the base mold and the paraffin block is ready to be cut.

Hematoxylin eosin (HE) staining plays an important role in tissue-based diagnosis. The pre-prepared paraffin blocks were mounted on a microtome and cut into thin slices. The slices are attached to the microscope slide then the first staining step begins with de-waxing using a solvent to remove wax from the slides prior to staining. This is always done as part of the coloring process. HE staining uses two dyes, hematoxylin and eosin. This combination is used as a dye for different tissue elements. Hematoxylin is a basic dye of acidic elements that is basophilic to produce a purplish blue color, such as the structure of the cell nucleus (which contains DNA and nucleoproteins), and organelles containing RNA such as ribosomes and rough endoplasmic reticulum. Eosin is an acidic dye that colors the basic elements of the cell giving it a reddish or pink color. Eosin is a basic dye of basic elements that is acidophilic, covering the cytoplasm, cell walls, and extracellular fibers. After the coloring is complete, then it is covered with coverglass. This staining allows the anatomical pathologist to view tissue morphology (structure) and look for the presence of tumor cells under a microscope to assess viability.

IHC staining is used to improve accuracy in determining the number of viable cells because tumor necrosis assessed only by cell morphology is less accurate. In this study, a monoclonal antibody was used against the rough endoplasmic reticulum (rER) cytoskeleton-linking membrane protein P63 (CLIMP 63). CLIMP 63 is a rough endoplasmic reticulum (rER) that functions in protein transport and is very abundant in tumor cells. In tumor cells that undergo necrosis, rough endoplasmic reticulum (rER) changes, and disturbances in protein production.

This principle is used in the IHC staining technique using the monoclonal antibody CKAP4 where binding to the rough endoplasmic reticulum (rER) cytoskeleton-linking membrane protein P63 (CLIMP 63) will give a positive result. In viable cells, the cytoplasm contains many ribosomes and rough endoplasmic reticulum (rER) which will bind to IHC dye and give color to the cytoplasm, while necrotic cells will have a clear cytoplasm. Next, the counting process was carried out using a light microscope to count live (stained) and dead (unstained) cells. The percentage of viable cells was obtained by comparing the number of clear cells (unstained) to the total number of cells.

The collected data will be analyzed statistically using the SPSS 23 program. This study obtained quantitative data. To test whether there were differences between the seven groups, the Kruskall-Wallisdan Mann-Whitney test was used. Data is displayed in the form of tables and graphs.

III. RESULTS

Comparison of the viability of tumor tissue cells in vitro on HE staining with descriptive and analytical analysis in the form of ANOVA test with alternative Kruskal Wallis test. The value of tumor cell
viability on HE staining after exposure to nitrogen for 10 minutes, 15 minutes, 20 minutes, 25 minutes, 30 minutes and controls, respectively 53.57 ± 28.68; 33.57 ± 26.57; 26.25 ± 27.5; 2.14 ± 2.67; 2.14 ± 3.94 and 79.38 ± 19.17. These results indicate the viability of tumor cells by HE staining in each group.

Meanwhile, the calculation of the distribution of tumor cell viability on HE staining using the non-parametric Shapiro-Wilk test shows an abnormal distribution for tumor cell viability on HE staining (p < 0.05) in both groups, namely exposure time of 25 minutes, and 30 minutes. minute. Therefore, the Kruskal Wallis non-parametric test was performed to statistically analyze the differences between each appropriate variable.

Based on the analysis of the Kruskal Wallis test, this study found a significant difference between the mean viability of tumor cells on HE staining for each group (p=0.000). This shows that the viability of tumor cells on HE staining in each group with exposure time of 10 minutes, 15 minutes, 20 minutes, 25 minutes, 30 minutes and the control group there is a significant difference.

Table 1. Analysis of the Kruskal Wallis test for tumor cell viability on HE staining of each group

<table>
<thead>
<tr>
<th>ExposureTime</th>
<th>N</th>
<th>Percentage</th>
<th>Mean</th>
<th>SD</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viability Tumor Cells</td>
<td>10 Min</td>
<td>7</td>
<td>87.5</td>
<td>53.57</td>
<td>28.68</td>
</tr>
<tr>
<td></td>
<td>15 Min</td>
<td>7</td>
<td>87.5</td>
<td>33.57</td>
<td>26.57</td>
</tr>
<tr>
<td></td>
<td>20 Min</td>
<td>4</td>
<td>50</td>
<td>26.25</td>
<td>27.5</td>
</tr>
<tr>
<td></td>
<td>25 Min</td>
<td>7</td>
<td>87.5</td>
<td>2.14</td>
<td>2.67</td>
</tr>
<tr>
<td></td>
<td>30 Min</td>
<td>7</td>
<td>87.5</td>
<td>2.14</td>
<td>3.94</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>100</td>
<td>79.38</td>
<td>19.17</td>
<td></td>
</tr>
</tbody>
</table>

Based on the Kruskal-Wallis test above, to find out which groups have significant differences between other groups requires a post hoc analysis with the Mann-Whitney test and independent sample t-test. In this study, there were significant differences in the comparison between the control group and the 15-minute group (p=0.002); control group with 20 minutes group (p=0.003); control group with 25 minutes group (p=0.001); control group with 30 minutes group (p=0.001); group of 10 minutes with a group of 25 minutes (p=0.010); the 10-minute group with the 30-minute group (p=0.008); group of 15 minutes with a group of 25 minutes (p=0.010); and the 15-minute group with the 30-minute group (p=0.009)

Table 2. Post Hoc Analysis of the comparison of tumor cell viability on HE staining of each group

<table>
<thead>
<tr>
<th>Exposure</th>
<th>10 Min</th>
<th>15 Min</th>
<th>20 Min</th>
<th>25 Min30 Min</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>0.201a</td>
<td>0.158a</td>
<td>0.010a</td>
<td>0.008a</td>
<td>0.058a</td>
</tr>
<tr>
<td>15 Min</td>
<td>0.201a</td>
<td>0.674a</td>
<td>0.010a</td>
<td>0.009a</td>
<td>0.002a</td>
</tr>
<tr>
<td>20 Min</td>
<td>0.158a</td>
<td>0.674a</td>
<td>0.126a</td>
<td>0.097a</td>
<td>0.003a</td>
</tr>
</tbody>
</table>
Comparison of the viability of tumor tissue cells in vitro on IHC staining with descriptive and analytical analysis in the form of ANOVA test with alternative Kruskal Wallis test. The value of tumor cell viability on IHC staining after exposure to nitrogen for 10 minutes, 15 minutes, 20 minutes, 25 minutes, 30 minutes and controls respectively 33.33 ± 38.19; 3.33 ± 5.77; 000 ± 000; 000 ± 000; 000 ± 000 and 86.67 ± 11.55. These results indicate the viability of tumor cells by IHC staining in each group.

The calculation of the distribution of tumor cell viability on IHC staining using the non-parametric Shapiro-Wilk test shows an abnormal distribution for tumor cell viability on IHC staining (p < 0.05) in both groups, namely the exposure time of 15 minutes, and the group control. Meanwhile, the exposure time groups of 20 minutes, 25 minutes, and 30 minutes were not normally distributed because they had a value of 0. Therefore, the Kruskal Wallis non-parametric test was carried out to statistically analyze the differences between each appropriate variable.

Based on the analysis of the Kruskal Wallis test, in this study a significant difference was found between the mean tumor cell viability on IHC staining for each group (p=0.020). This shows that the viability of tumor cells on IHC staining in each group with exposure time of 10 minutes, 15 minutes, 20 minutes, 25 minutes, 30 minutes and the control group there is a significant difference.

<table>
<thead>
<tr>
<th>Exposure Time</th>
<th>N</th>
<th>Percentage</th>
<th>Mean</th>
<th>SD</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 Min</td>
<td>3</td>
<td>100</td>
<td>33.33</td>
<td>38.19</td>
<td>0.020</td>
</tr>
<tr>
<td>15 Min</td>
<td>3</td>
<td>100</td>
<td>3.333</td>
<td>5.77</td>
<td></td>
</tr>
<tr>
<td>20 Min</td>
<td>3</td>
<td>100</td>
<td>000</td>
<td>000</td>
<td></td>
</tr>
<tr>
<td>25 Min</td>
<td>3</td>
<td>100</td>
<td>000</td>
<td>000</td>
<td></td>
</tr>
<tr>
<td>30 Min</td>
<td>3</td>
<td>100</td>
<td>000</td>
<td>000</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>100</td>
<td>86.67</td>
<td>11.55</td>
<td></td>
</tr>
</tbody>
</table>

Based on the Kruskal-Wallis test above, to find out which groups have significant differences between other groups requires a post hoc analysis with the Mann-Whitney test. Based on table 4, in this study, there were significant differences in the comparison between the control group and the 10-minute group (p=0.046), the control group and the 15-minute group (p=0.043), the control group and the 20-minute group (p=0.034), the control group with a group of 25 minutes (p=0.034), the control group with a group of 30 minutes (p=0.034).

Table 4. Post Hoc Analysis of Comparison of tumor cell viability on CKAP4 IHC staining of each group

<table>
<thead>
<tr>
<th>Exposure Time</th>
<th>N</th>
<th>10 Min</th>
<th>15 Min</th>
<th>20 Min</th>
<th>25 Min</th>
<th>30 Min</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 Min</td>
<td></td>
<td>0.246</td>
<td>0.121^a</td>
<td>0.121^a</td>
<td>0.121^a</td>
<td>0.046^a</td>
<td></td>
</tr>
</tbody>
</table>
IV. DISCUSSION

The use of cryosurgery in the treatment of bone tumors was first performed at the Memorial Sloan-Kettering Cancer Center in the United States in 1964 as a palliative procedure in patients with lung metastatic humeral bone tumors (RALPH C Marcove, 1982; Ralph C Marcove & Miller, 1969). In 1984, the use of liquid nitrogen was introduced as the main treatment of lesions in osteosarcoma (R C Marcove, 1984). The use of cryosurgery was found to selectively damage tissues which was regulated by alternating cooling and thawing (Goldstein & Hess, 1977).

In cryosurgery, cell damage occurs through two mechanisms, namely direct injury to cells and the second is intracellular ice formation. There are two hypotheses of direct injury to cells, the first is the 'minimum volume' hypothesis. Cells will shrink at low temperatures and if this condition continues then the intracellular compartment will lose its compensatory ability so that the concentration inside the cell increases. During the thawing process, the content inside the cell is higher than outside the cell so that the administration of hypotonic fluid will cause the cell to burst due to the osmosis process. The second hypothesis is the destabilization of cell membranes during the freezing and thawing processes. When the cell is given a temperature of less than -10°C, about 90% of the water in the cell will come out by osmosis. However, when the cell undergoes thawing, water does not osmosis into the cell and the cell does not expand again. In the second mechanism, there is ice formation in the cells as a result of exposure to cells in an environment below freezing point for a long time. Formation of ice in this cell will make the cell die (Yiuetal, 2007).

The advantages of cryosurgery are short processing times, minimal pain, and low risk of bleeding which will also contribute to a reduced period of anesthesia required and length of hospital stay (Goldstein & Hess, 1977). This makes cryosurgery an attractive therapeutic option in tumor management (H Tsuchiya et al., 2005). In this study, the viability of tumor cells was analyzed after cryosurgery based on hematoxylin-eosin staining and immunohistochemistry. The immunohistochemical examination assessed was CKAP4. CKAP4 is a palmitoylated type transmembrane protein found in the endoplasmic reticulum (ER) (Harada et al., 2020). CKAP4 has a role in promoting the proliferation of normal cells and tumor cells (Hirokazu Kimura et al., 2016). In a study by Han it was found that CKAP4 expression is negatively associated with the prognosis of patients with pancreatic and lung cancer (Han et al., 2015).

In this study, the tissue used was a piece of osteosarcoma tissue from the amputate. There were 5 treatment groups in osteosarcoma tissue that were treated with cryosurgery, namely the osteosarcoma group with 10 minutes of time, the osteosarcoma group with 15 minutes of time, the osteosarcoma group with 20 minutes of cryosurgery, the osteosarcoma group with 25 minutes of cryosurgery treatment, and osteosarcoma group with cryosurgery treatment for 30 minutes.

In the results of this study the 10 and 15 minute groups still obtained tumor viability with results of 30.5% and 12.25% respectively on staining. Hematoxylin Eosin and 24.9% and 9.6% on immunohistochemical examination. Tumor viability was not obtained or 0% in the 20-, 25- and 30-min groups on Hematoxylin Eosin staining and on staining immunohistochemistry.

This is in accordance with a study conducted by Tsuchiya, the cryosurgery procedure used was freezing for 20 minutes with promising results (H Tsuchiya et al., 2005). This procedure has been used in many other studies with the same results (Igarashi et al., 2014; Hiroaki Kimura et al., 2016; Hiroyuki Tsuchiya et al., 2010; Xu et al., 2020). In Xu's study, the use of a freezing time of 20 minutes resulted in good function and provided biomechanical stability as found in prostheses (Xu et al., 2020). In Kimura's study, the success rate was 87.5%,
where there was only one patient who failed, and recommends using this method because of its low complication rate (Hiroaki Kimura et al., 2016). All of these data were obtained on a procedure duration of 20 minutes whereas the data that could be found on cryosurgery using different time durations was very limited. This makes the results obtained from this study very useful for increasing understanding of cryosurgery.

V. CONCLUSION

In this study, at 15 minutes of exposure, a significant decrease in tumor cell viability was found compared to the control group on HE staining and tumor viability decreased with increasing exposure time. At 10 minutes of exposure, a significant decrease in tumor cell viability was found compared to the control group on CKP4 IHC staining and tumor viability decreased with increasing exposure time. At the 20th minute to the 30th minute exposure, no tumor cells were found on CKP4 IHC staining. The best minimum time for cryosurgery for primary bone tumor cell therapy is 20 minutes.

REFERENCE