RESEARCH ARTICLE

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Morphological Changes and Apoptosis of Buccal Mucosa Basal Epithelium in Heads and Necks during Cancer Radiotherapy

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ABSTRACT

Background: Radiotherapy is generally used to treat head and neck malignancy through high radiation, focusing on killing cancer cells. However, some adverse effects in oral mucosal tissue, including cell morphology changes and apoptosis, are commonly found. This study aims to determine the morphological changes and apoptosis of buccal mucosa epithelium after radiotherapy in head and neck cancer (HNC) patients.

Methods: This study involved 8 subjects of patients diagnosed with HNC. Buccal mucosal smear samples were collected using cytobrush two times, before and after radiotherapy, with a dose of 70 Gy in 7 weeks. The specimens were prepared and stained using Feulgen and Rosenbeck technique. Observations were made using a light microscope with a count per 1000 epithelial cells. Statistical analysis was performed using statistical software with Pearson's correlation test and significance t-test ($p < 0.05$) between irradiated and non-irradiated samples.

Results: Data analysis showed significant changes in cell morphological damage and apoptosis in patients before and after radiotherapy. It increases in the number of micronuclei ($p = 0.001$), broken egg ($p = 0.001$), binuclei ($p = 0.003$), pyknosis ($p = 0.033$), karyorrhexis ($p = 0.020$), and karyolysis ($p = 0,004$).

Conclusions: The effects of radiation reflect morphological changes and apoptosis in the buccal mucosa basal epithelium in HNC patients.

INTRODUCTION

Cancer is a malignancy caused by the uncontrolled growth of cells or tissue abnormalities to form a mass [1,2]. Multiple factors cause cancer, such as carcinogenesis, genetics, and viral infections. One type of cancer contributing to a high incidence and mortality in Indonesia is head and neck cancer (HNC). Based on Riskesdas (Baseline Health Research) data, HNC is a malignancy in the fourth rank and is found more in men than women [3-5]. The high incidence of HNC is closely related to excessive alcohol and tobacco consumption. Besides, immunological factors, viral infections such as human papillomavirus and Epstein Barr Virus (EBV), and genetic factors contributed to HNC development [6].

The HNC treatment approach is usually conducted with chemotherapy and radiotherapy. Radiotherapy is a neoadjuvant, primary, or adjuvant for surgical therapy and chemotherapy in more than 50% of HNC patients by focusing high radiation energy on the cancer tissue. The high success rate of radiotherapy cures many malignancies when treated early [2,7,8]. However, long-term therapy with high doses can have side effects. One of the common side effects of head and neck radiotherapy is oral mucositis. High-dose exposure to radiotherapy can also lead to genotoxicity leading to high levels of cell damage and

apoptosis [9]. However, there have not been many reports regarding changes in morphology and cell apoptosis on radiotherapy at a dose of 70 Gy in HNC patients.

This study aims to examine the morphological changes and apoptosis of buccal mucosa epithelium post-radiotherapy at a dose of 70 Gy in HNC patients.

METHODS

Sample Collection

This study was conducted with a retrospective design. The observational study involved HNC patients who had been diagnosed by proper clinical histopathology examinations and undergone radiotherapy at Prof. Dr. Margono Soekarjo Hospital, Purwokerto, with an age range less than 60 years old, and did not have habits of smoking and/ or alcohol consumption. The ethical approval was obtained from The Health Research Ethics Committee of Dr. Moewardi Hospital, Surakarta (No.36/I/HREC/2017).

Eight subjects were enrolled in the study and signed informed consent. Specimens of buccal mucosa swabs were collected from subjects before radiotherapy and post-radiotherapy after final irradiation at a dose of 70 Gy within seven weeks. The specimens were collected using a smear method on the superficial epithelium of the mucosa using cytobrush [10]. The staining of the preparations was carried out using the Feulgen and Rosenbeck method with Schiff's reagent and fast green 1% staining.

Assessment of Morphological Changes and Apoptosis Cell

Observations were conducted by three observers using an optic-lab Pro microscope and Image Raster software to determine the number of observed cells between specimens before and after radiotherapy. The nuclear morphological changes such as micronucleus, binuclei, and broken eggs were calculated to calculate the apoptosis equation from the four forms, namely pyknosis, karyorrhexis, and karyolysis [11].

The calculation criteria are identified by adding additional nuclei with a maximum diameter of one-third of the nucleus with a texture and color resembling the nucleus. Binuclei, a broken egg, is indicated by the criteria of having two nuclei of the same large cell, and the two nuclei are closely spaced. The central nucleus, the shoots, and the coloring part are used as the broken egg criteria indicators. Dense, even, and color, a shrunk core indicates a pyknotic marker. Karyorrhexis is calculated as cells with nuclei that experience disintegration. The nucleus begins to disappear or experience decay of a dense speckled nucleus pattern showing nuclear fragmentation leading to the overall decay. Karyolysis was observed using Periodic Acid Schiff and Fast Green staining. The count was cells with nuclei that did not contain chromosomes, which looked like ghost images.

Data Analysis

Analysis of the mean cell morphological damage and apoptosis was performed to determine the impact of radiotherapy on HNC patients. The analysis of the validity of the observer was conducted through the validity test and Pearson correlation. The analysis shows a significance value of $p < 0.0001$ among each observer to determine the level of validity among observers. Mean comparisons of morphological damage and apoptotic results were performed using a paired t-test between patients before and after therapy. A significant difference is indicated by the p-value < 0.05.

RESULTS

Sample Characteristics

This study was conducted by involving eight patients who had been diagnosed with HNC. The characteristics of the research subject can be seen in **Table 1**.

The buccal mucosa smear was carried out two times before irradiation and after irradiation with a dose of 70 Gy within seven weeks. An illustration of the cytological smear results of the buccal mucosal smear can be seen in **Figure 1**.

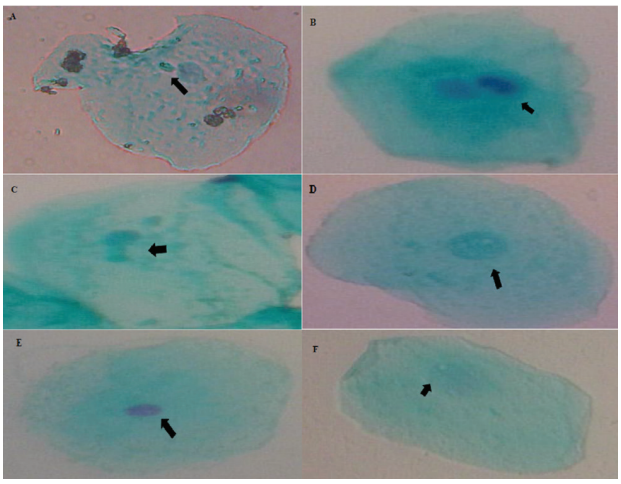

Figure 1. Morphological damage *(arrows)* of buccal mucosal smear stained with Schiff acid and fast green at 400x magnification; (A)micronucleus; (B) Binucleus; (C) Broken egg; (D) Picnosis cell; (E) karyorrhexis; (F) karyolysis.

Data Analysis

Analysis of the mean changes in cell morphological damage and apoptosis can be seen in **Table 2**. The results showed increased damage to cell morphology and apoptosis post-radiotherapy, indicated by an increase in micronucleus, binuclei, broken egg, pyknosis, karyorrhexis, and karyolysis with SD ranging from 0-1.

The analysis results (**Table 3**) showed a significant change with a p-value < 0.05 in all variables: micronucleus, broken egg, binuclei, pyknosis, karyorrhexis, and karyolysis in patients after receiving radiotherapy.

Table 1. The characteristic of subjects with head and neck cancer who received radiotherapy

No	Sex	Age	Head and neck cancer types	Frequency of chemotherapy
1.	Female	30	Nasopharyngeal Cancer	6 times
2.	Female	21	Squamous Cell Neck Cancer	6 times
3.	Female	26	Nasopharyngeal Cancer	6 times
4.	Female	32	Paranasal Sinus Cancer	5 times
5.	Male	49	Nasopharyngeal Cancer	6 times
6.	Male	32	Nasopharyngeal Cancer	6 times
7.	Male	51	Squamous Cell Tongue Cancer	8 times
8.	Male	57	Paranasal Sinus Cancer	5 times

Table 2. Mean and standard deviation of cell morphological damage and apoptosis before and after radiotherapy at a dose of 70 Gy within seven weeks

No	Variables	Groups	n	Mean	SD
1.	Micronucleus	Before radiotherapy	8	4.208	0.532
		After radiotherapy		7.166	1.553
2.	Broken egg	Before radiotherapy	8	2.958	0.602
		After radiotherapy		5.333	1.247
3.	Binuclei	Before radiotherapy	8	2.041	0.756
		After radiotherapy		4.041	0.575
4.	Pyknosis	Before radiotherapy	8	3.041	0.916
		After radiotherapy		4.291	0.950
5.	Karyorrhexis	Before radiotherapy	8	1.833	0.590
		After radiotherapy		3.041	0.744
6.	Karyolysis	Before radiotherapy	8	1.750	0.660
		After radiotherapy		2.958	0.653

SD = Standard Deviation

Table 3. Results of comparative analysis mean number of morphological damage and cell apoptosis of patients before and after radiotherapy at a dose of 70 Gy within seven weeks

No	Variables	Groups	t-count	p-value
1.	Micronucleus	Before radiotherapy After radiotherapy	5.596	0.001
2.	Broken egg	Before radiotherapy After radiotherapy	5.445	0.001
3.	Binuclei	Before radiotherapy After radiotherapy	4.341	0.003
4.	Pyknosis	Before radiotherapy After radiotherapy	2.658	0.003
5.	Karyorrhexis	Before radiotherapy After radiotherapy	2.998	0.020
6.	Karyolysis	Before radiotherapy After radiotherapy	4.192	0.004

SD = Standard Deviation

DISCUSSION

Head and neck cancer (HNC) is a malignancy caused by the body's new tissue due to uncontrolled cell growth in the head and neck [8,12]. Surgery, chemotherapy, and radiotherapy are primary therapies performed on patients with HNC. Treatment with chemotherapy and radiotherapy is the most robust approach in patients with HNC [13-16]. The anatomical location, size, and clinical symptoms of HNC are not specific, making it difficult to carry out the biopsy or early detection [17-19]. However, there are still many challenges and effects of this treatment that require a more comprehensive study. Chemotherapy uses cytotoxic drugs orally or intravenously to kill cancer cells. In addition, radiotherapy is carried out using definitive radiotherapy at a dose of 66-70 Gy [20].

Radiotherapy is carried out by utilizing ionization from the flow of photons and charged particles, thereby interacting with DNA molecules that result in cell death,

induction of mutations, and genetic and chromosomal instability [21-23]. However, radiotherapy gives side effects to patients such as loss of taste, changes in composition and volume of saliva, xerostomia, fungal infections, caries, periodontal tissue damage, trismus, mucositis, and osteoradionecrosis [24-26]. Morphological changes and cell apoptosis after radiotherapy are found. The increase of micronucleus due to cellular and chromosome instability was reported as a significant effect of 20 Gy radiation. After receiving radiation, the other effect of morphological changes is an increase in broken eggs. A previous study showed that broken eggs could also be induced by radiation from electromagnetics on the telephone [27]. The increase in radiotherapy-induced binuclei is due to the genotoxic effect on the mucosal epithelium, which indicates cancer formation.

Increased apoptosis was shown after ionizing radiation via internal or mitochondrial pathways [28,29]. The cell apoptosis via the mitochondrial pathway is regulated by the Bcl-2 protein complex present on the outer surface of the mitochondria. When DNA damage occurs, the p53 gene activation process will activate the Bcl-2 protein complex via Bac, Bad, and Bid. This interaction causes Bcl-2 to release the apoptotic protease activating factor-1 (apaf-1), which is followed by the normal function of the anti-apoptosis being disrupted [30]. In addition, the Bax-Bcl-2 interaction causes pro-caspase-3 to activate proteolytic digestion of protein structures in the cytoplasm, digestion of DNA into phases of 3 'OH ends, and various morphological changes in apoptotic cells [31-33]. Morphological and apoptotic damage can be seen from pyknosis, karyorrhexis, and karyolysis. Cell damage happens with increased amounts of genotoxic apoptosis. Damage to DNA molecules induces programmed cell death (apoptosis) or genetic instability and oncogenic mutations [34,35].

The DNA damage and apoptosis of the buccal mucosa epithelial cells characterizes injury of mucosa membranes, as the initial phase of oral mucositis pathogenesis caused by radiotherapy and/or chemotherapy. The apoptotic cells will consequently release endogenous damage-associated pattern molecules (DAMPs) which trigger the inflammation cascades that lead to ulceration [36]. An increase of apoptotic and inflammation markers expressions, namely p53, BCl-2, MCl-1, TNF, and IL-1β, was found in cytological smear examination of HNC patients who developed oral mucositis during radiotherapy [37]. Oral mucositis causes pain and difficulty to eat which may compromise the nutrition status, quality of life, as well as cancer treatment of HNC patients. This study depicted cellular changes of buccal mucosa epithelium in the early stage of radiotherapy at a dose of 70 Gy in NHC patients. Therefore, some mucositis prevention or treatments are beneficial to be administered along with radiotherapy, for example, early nutritional intervention, antioxidant agents, benzydamine hydrochloride mouth rinse, photobiomodulation, etc, to improve the success of radiotherapy in HNC patients.

CONCLUSIONS

Morphological changes and apoptosis of buccal mucosa epithelium are found in patients with HNC after radiotherapy, indicated by an increase of micronucleus, broken egg, binuclei, pyknosis, karyorrhexis, and significant karyolysis in buccal mucosa epithelium.

DECLARATIONS

Ethics Approval

The authors were given ethical clearance by Universitas Sumatera Utara Research Committee for this research. The necessary document is provided in a separate file. The ethical clearance letter number is 195/KEP/USU/2020.

Conflict of Interest

The authors declared there is no competing interest in this study.

Acknowledgment

HBW, AW, DV, DJW, and TW designed the study. NPB and CCP carried out the laboratory work. CCP, NPB, HBW, and TW analyzed the data. CCP, NPB, HBW, and TW wrote the manuscript. All authors read and approved the final version of the manuscript.

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