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Effect of short-term fasting on the cisplatin activity in human oral squamous cell carcinoma cell line HN5 and chemotherapy side effects

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Abstract

Background Ketogenic interventions like short-term fasting show potential as complementary therapies to enhance the effectiveness of chemotherapy for cancer. However, the specific effects of fasting on head and neck squamous cell carcinoma (HNSCC) cells and healthy oral mucosa cells during these treatments are not well understood. This study investigates whether short-term fasting can differentially impact HNSCC cell survival and viability compared to healthy keratinocytes while undergoing standard chemotherapy regimens.

Methods This study investigated the effects of fasting on cell viability in HN5 cell line and healthy oral keratinocyte cells. The HN5 cell line, derived from human tongue squamous cell carcinoma, and primary human keratinocytes isolated from the basal layer of gingival epithelium were divided into three groups: (1) control, (2) treated with the standard chemotherapeutic agent cisplatin, and (3) treated with cisplatin under fasting conditions achieved through 48-hour glucose restriction mimicking the blood glucose levels of fasted individuals. Cell proliferation was assessed at 48 and 72 h using the MTT assay, a colorimetric method based on mitochondrial dehydrogenase activity. Flow cytometry analysis with specific apoptosis and necrosis markers distinguished between early and late apoptotic, necrotic, and viable cells.

Results Cell viability in HN5 and healthy keratinocyte cells decreased in cisplatin with low glucose groups compared to cisplatin and control groups. The same results were observed for healthy keratinocyte cells; only a decrease in cell viability in cisplatin groups compared to control groups was observed, which was not statistically significant. Cell apoptosis in HN5 and healthy keratinocyte cells increased in cisplatin with low glucose groups compared to cisplatin and control groups. In healthy keratinocyte cells, the cisplatin with low glucose group showed an impressive increase in necrosis, late apoptosis, and early apoptosis and a significant decrease in live cells compared with other groups.

Conclusion This study revealed that short-term fasting chemotherapy significantly improved HNSCC cell line apoptosis and necrosis.

Keywords Fasting, Squamous cell carcinoma of head and neck, Keratinocytes, Apoptosis, Necrosis

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Introduction

The prevalence of cancer is increasing worldwide, bringing considerable costs to healthcare systems. Cancer is the second leading cause of death worldwide [1]. Head and neck SCC is the seventh most common cancer diagnosis worldwide [2]. Tobacco use, excessive alcohol consumption, human papillomavirus (HPV) infection, betel quid chewing, and poor diet are well-established risk factors for oral cancer development [3–5]. Notably, modern diets, often high in refined carbohydrates, have been implicated in carcinogenesis [6]. Conversely, the ketogenic diet, which restricts carbohydrates and promotes fat metabolism, may create an unfavorable microenvironment for cancer cells [7].

Fasting, particularly short-term fasting (STF), has emerged as a promising strategy to enhance cancer treatment outcomes [8]. Studies suggest fasting can augment cancer cell sensitivity to chemotherapy while protecting healthy cells [9, 10]. This effect is likely attributed to a metabolic shift in cancer cells towards less efficient energy production pathways than healthy cells [11]. The use of conventional treatments, including surgery, radiotherapy, and chemotherapy drugs, remains the most accepted approach among physicians and patients. This is due to their easy access, lower cost, and effectiveness against various cancers. While newer approaches like targeted therapy drugs hold promise and demonstrate effectiveness in cancer treatment [12, 13], their application is limited to specific cancer subgroups. Additionally, these therapies come with a high cost and pose access challenges [14, 15]. Chemotherapy and radiotherapy destroy cancer cells through genotoxicity, which is the production of reactive oxygen species (ROS) [16]. Healthy cells are also likely to be destroyed and severely damaged, causing side effects such as bone marrow suppression, fatigue, nausea, diarrhea, oral mucositis, and even death. Despite the various treatment modalities used for HNSCC, the survival rate for the disease remains relatively low, with a 5-year survival rate of 50–60% [17, 18].

Due to the limited effectiveness of common treatments caused by toxicity in healthy tissues, recent research has focused on developing adjuvant strategies utilizing chemoproteomic agents [19–21]. These agents exploit differential effects to selectively increase tumor cell sensitivity to treatment while enhancing the resistance of healthy cells to toxicity [22]. Most cancers, especially carcinomas of epithelial origin, appear to be caused by a disturbance in metabolism associated with the modern lifestyle [23]. Diets high in carbohydrates, a common feature of modern lifestyles, can induce epigenetic modifications through specific chromatin conformations and alterations in DNA structure. These epigenetic changes can impact genomic stability and the production of proteins and mRNA, potentially contributing to metabolic disorders [24]. Modern lifestyles have a significant impact on the development of breast, cervical, oral, and gastric cancers, among others [25]. Another aspect of cancer treatment is the use of the ketogenic diet as an adjuvant therapy. This diet likely creates an unfavorable metabolic environment for cancer cells, making it a promising candidate for patient-specific, multifactorial treatment approaches. Some cancer cells, due to mitochondrial dysfunction, lack the ability to metabolize ketone bodies produced by the ketogenic diet. Additionally, the diet reduces blood glucose levels, which can lead to a decrease in insulin and insulin-like growth factor (IGF), both of which are important drivers of cancer cell proliferation [26, 27]. A rapid increase in carbohydrate consumption plays a significant role in these metabolic changes and tumorigenesis [28]. Tumor tissue consumes significantly more glucose than healthy tissue. Even in the presence of oxygen (aerobic conditions), tumor cells produce large amounts of lactate. This is a key difference from healthy tissue, which minimizes lactate production through respiration [29]. Therefore, limiting or eliminating glucose sources through a ketogenic diet has been explored as a potential approach for cancer patients [30, 31]. Previous research supports the idea that ketogenic interventions, such as fasting, could be effective adjuncts to improve the outcomes of radiotherapy and chemotherapy [32–35]. Fasting enhances the sensitivity of cancer cells to chemotherapy during fasting [23]. This effect is likely due to a combined increase in ROS production and a decrease in adenosine triphosphate (ATP) levels, caused by a metabolic shift from glycolysis to mitochondrial metabolism in tumor cells [36]. During fasting, healthy cells undergo a metabolic switch from growth to maintenance mode. This involves a decrease in glucose and growth factor availability, accompanied by an increase in ketone bodies. Ketone bodies serve as an alternative fuel source and are associated with reduced cellular damage in healthy tissues [37]. The metabolic difference underlies the differential stress resistance phenomenon (DSRP) in healthy and cancer cells [38]. STF is a dietary approach that involves completely restricting calorie intake for a limited period of time. STF is considered a non-invasive and cost-effective complementary treatment option [39]. Several studies have shown that STF increases a specific type of cancer cell death, while also protecting healthy cells during chemotherapy. This effect has been observed in both laboratory in-vitro and in-vivo studies [40–46]. However, no study has evaluated the effects of fasting on head and neck squamous cell carcinoma (HNSCC) and healthy oral mucosa cells under chemotherapy. The available preclinical and limited clinical data suggest that intermittent fasting may have favorable effects on breast cancer outcomes [47]. Diabetic-FBG (fasting blood glucose) level was found to be an independent prognostic factor

for patients with oral cancer [48, 49]. Fasting or fasting-mimicking diets have shown potential in modulating the tumor biology and improving the effects of cancer therapies in brain tumors [50] but more robust clinical studies are needed to confirm these findings.

SCC is the most common malignant tumor of the head and neck [51]. It is associated with a low life expectancy and often shows a poor response to treatment, exhibiting high resistance. Surgery, radiotherapy, and chemotherapy (for distant metastasis) are the main treatment options for HNSCC [51]. In many cases, significant side effects of chemotherapy, such as oral mucositis, can disrupt the treatment process [52–55]. Therefore, it is necessary to identify methods that increase cancer cells' destruction while reducing conventional therapies' toxicity on healthy cells. Therefore, this study was designed to investigate the effect of STF on HNSCC cells and healthy keratinocyte cells under chemotherapy.

Methods

Ethical statement

The protocol of this experimental and interventional in-vitro study was approved by the Ethics Committee of Tehran University of Medical Sciences (ethical code: IR.TUMS.DENTISTRY.REC.1400.124) and Informed consent was obtained from all individuals. All methods were performed in accordance with the relevant guidelines and regulations and this study was conducted in accordance with the Declaration of Helsinki [56].

Cell culture

The HN5 cell line (code: NCBI, 30196) was purchased from the National Cell Bank of Iran, affiliated with the Pasteur Institute of Tehran, Iran. This cancer cell line was derived from a tongue squamous cell carcinoma in a 73-year-old man (Supplementary file 1). The cells were cultured in a Dulbecco's Modified Eagle Medium (DMEM) (BIOSERA, USA) that contains 10% Fetal Bovine Serum (FBS) (GIBCO, USA). They were cultured in a cellular incubator for proliferation to reach a suitable density at 37 °C and 5% CO_2 and 1-2% humidity for 24 h. Pen/strep (ATOCEL Company, Budapest), a solution containing standard antibiotics, penicillin, and streptomycin, was added to prevent the growth of a variety of Gram-positive and Gram-negative bacteria.

Human healthy keratinocyte cell was obtained from gingival tissue removed under sterile conditions for crown lengthening, then quickly transferred to the laboratory in a phosphate-buffered saline (PBS) container along with 3% pen/strep antibiotic in the vicinity of a 4 °C ice pack. The sample was washed several times with hank buffers containing antibiotics in the laboratory and under sterile conditions below the hood, then the target tissue was placed in tissue solvent solution including DMEM (BIOSERA, USA) and collagenase type 1 (Worthington Biochem, Freehold, NJ, USA) with concentration 250 U/ml and was incubated in a shaking incubator for 1 to 2 h at 37 °C and 5% CO_2 for tissue digestion. After the tissue was digested, filtration was performed with 70 µm filters (Falcon, BDL labware, Franklin Lakes, NJ, USA) to remove undigested tissue fragments and impurities. Afterward, the solution containing the sample was centrifuged in at 1500 rpm for 5 minutes to form a cell plate. Then, the cells were cultured in a T75 flask (Greiner, Frickenhausen, Germany) containing DMEM 80%/ F12 medium (BioChrom AG, Berlin, Germany) with 10% FBS (GIBCO, USA) and 10% pen/strep. The culture medium has been changed every two days. And each week it's got it-term trypsinization for 5 minutes by 1 mL of Trypsin-EDTA (Sigma-German) solution (Trypsin 0.25% and one molar EDTA), the cells got separated and cultured in a new flask with a density of 1×10^5 cells/cm^2.

After Trypsinization of the cells and centrifuging and suspending the cell's sediment in one milliliter of culture medium, cell counting was done. Then, according to the number of cells obtained in a volume of 1000 µl, using a simple ratio, a volume of suspension containing 5000 cells was calculated. According to the calculations, 5000 cells were cultured in each well of a 96-well plate (in 3 groups of 3 replicates) and the cells were placed in an incubator for 24 h.

SCC cells are divided into three groups; Cells without treatment (SCC-1), under treatment with a standard chemotherapeutic agent (SCC-2), and cells under treatment with standard a chemotherapeutic agent and fasting condition (SCC-3). Healthy oral keratinocyte cells are divided into three groups; Cells without treatment (Healthy-1), under treatment with a standard chemotherapeutic agent (Healthy-2), and cells under treatment with standard chemotherapeutic agent and fasting condition (Healthy-3).

In vitro fasting (short-term fasting)

Cellular fasting was done by glucose restriction to achieve blood glucose levels typical of fasted and healthy cells; for human cell lines, cells were washed twice with PBS before changing to a fasting medium. For Short-Term fasting medium (STF), cells were grown in DMEM medium without glucose (DMEM no glucose, Life Technologies, Cat. No. 11966025) supplemented with 0.5 g/L glucose (Sigma-Aldrich, Cat. No. G8769) and 1% FBS. For mimicking normal conditions, cells were grown in a DMEM medium without glucose (DMEM no glucose, Life Technologies, Cat. No. 11966025) supplemented with 1 g/L glucose (Sigma-Aldrich, Cat. No. G8769) and 10% FBS, referred to as control medium (CTR) [57]. All treatments were perform at 37 °C under 5% CO_2.

Forty-eight hours later, a chemotherapeutic agent was added to culture media.

Chemotherapeutic agent preparation

10 mg/mL vials of 1 mg/mL cisplatin were purchased from Mylan Company, France. In-vitro chemotherapy was performed by treating cells in a medium containing cisplatin with a dose of 8 µl/ml for 48 h.

Cell proliferation assay

After 48 and 72 h, cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) test. MTT powder was combined with PBS medium, and the resultant solution was applied to the wells of a 96-well microplate that contained cell lines and groups. After a four-hour incubation at 37 degrees Celsius, dimethyl sulfoxide was supplied to the wells, and the absorption rate was recorded at 570 nanometers.

Annexin V-FITC/PI staining and flow cytometry

After treatment with fasting and cisplatin alone or in combination, the cells were trypsinized and incubated with Annexin V–conjugated MicroBeads (Miltenyi Biotec GmbH, Germany), according to the manufacturers protocol. The apoptosis rate was then assessed using a FACS Calibur flow cytometer (BioTed USA), and the data was analyzed using Cell Quest (BD Biosciences) and FlowJo (Tree Star Inc., Ashland, OR, USA) software. According to the staining profile, the early apoptotic cells (FITC+ / PI), late apoptotic cells (FITC /PI+), necrotic cells (FITC+ /PI+), and intact cells (FITC /PI) were discriminated.

Statistical analysis

The data were analyzed using SPSS (version 22.0; SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8.2.1 (GraphPad Software, San Diego, CA). One-way ANOVA was used to compare the means of different groups, followed by Tukey HSD post hoc analysis for pairwise comparisons. A p-value (p) less than 0.05 was considered statistically significant.

Results

Cell viability in HN5 and healthy keratinocyte cells

Based on the 48 and 72-hour MTT analysis, the control groups (SCC-1 and Healthy-1) exhibited the highest cell viability (100%) for both HN5 and healthy keratinocyte cells. Cell viability was followed by the cisplatin-treated groups (SCC-2 and Healthy-2). The lowest average cell viability was observed in the groups treated with cisplatin under low glucose conditions (SCC-3 and Healthy-3), (Supplementary file 2 and 3) (Tables 1 and 2).

One-way ANOVA analysis revealed a statistically significant difference ($p<0.05$) in cell viability between groups for both HN5 and healthy keratinocyte cells at both time points. Specifically, in HN5 cells, significant differences were observed at 48 h ($p<0.001$, F=87.764) and 72 h ($p<0.001$, F=88.793) of MTT assay. Similarly, significant differences were found in healthy keratinocytes at 48 h ($p=0.002$, F=20.629) and 72 h ($p<0.001$, F=53.213) of MTT assay.

Tukey's HSD analysis in HN5 cells showed a statistically significant decrease in cell viability in cisplatin groups (SCC-2) ($p<0.05$) and cisplatin with low glucose groups (SCC-3) ($p<0.05$) to control groups (SCC-1). Also, the decrease in cell viability in the cisplatin with low glucose groups (SCC-3) to cisplatin groups (SCC-2) was statistically significant (Table 1; Fig. 1-A) ($p<0.05$). These results were found in both 48 and 72 h of MTT assay. These same results were also reported for the healthy keratinocyte cells; only a decrease in cell viability in cisplatin groups (Healthy-2) to control groups Healthy-1)

Table 1 Tukey HSD for MTT assay of cell viability HN5 cell line

Time of treatment	Cell type and sequence of treatment		Mean difference	Standard error	*p* value	95% Confidence interval for mean	
						Lower bound	Upper bound
48 h	HN5	Cisplatin	34.600	3.853	0.000 *	22.778	46.423
		Cisplatin + low glucose	49.806	3.853	0.000 *	37.983	61.629
	Cisplatin	HN5	-34.600	3.853	0.000 *	-46.423	-22.778
		Cisplatin + low glucose	15.206	3.853	0.018 *	3.3830	27.028
	Cisplatin + low glucose	HN5	-49.806	3.853	0.000 *	-61.629	-37.983
		Cisplatin	-15.206	3.853	0.018 *	-27.028	-3.383
72 h	HN5	Cisplatin	32.501	4.625	0.001 *	18.311	46.692
		Cisplatin + low glucose	61.599	4.625	0.000 *	47.409	75.790
	Cisplatin	HN5	-32.501	4.625	0.001 *	-46.692	-18.311
		Cisplatin + low glucose	29.098	4.625	0.002 *	14.908	43.288
	Cisplatin + low glucose	HN5	-61.599	4.625	0.000 *	-75.790	-47.409
		Cisplatin	-29.098	4.625	0.002 *	-43.288	-14.908

*: *p* value ≤ 0.05

Table 2 Tukey HSD for MTT assay of cell viability healthy oral keratinocyte

Time of treatment	Cells and sequence of treatment		Mean difference	Standard error	*p* value	95% confidence interval for mean	
						Lower bound	Upper bound
48 h	healthy	Cisplatin	6.002	4.443	0.421	-7.631	19.636
		Cisplatin + low glucose	27.166	4.443	0.002 *	13.532	40.799
	Cisplatin	healthy	-6.002	4.443	0.421	-19.636	7.631
		Cisplatin + low glucose	21.163	4.443	0.007 *	7.530	34.797
	Cisplatin + low glucose	healthy	-27.166	4.443	0.002 *	-40.799	-13.532
		Cisplatin	-21.163	4.443	0.007 *	-34.797	-7.530
72 h	healthy	Cisplatin	15.361	4.493	0.033 *	1.5757	29.146
		Cisplatin + low glucose	45.551	4.493	0.000 *	31.766	59.336
	Cisplatin	healthy	-15.361	4.493	0.033 *	-29.146	-1.576
		Cisplatin + low glucose	30.190	4.493	0.001 *	16.405	43.975
	Cisplatin + low glucose	healthy	-45.551	4.493	0.000 *	-59.336	-31.766
		Cisplatin	-30.190	4.493	0.001 *	-43.975	-16.405

*: *p* value ≤ 0.05

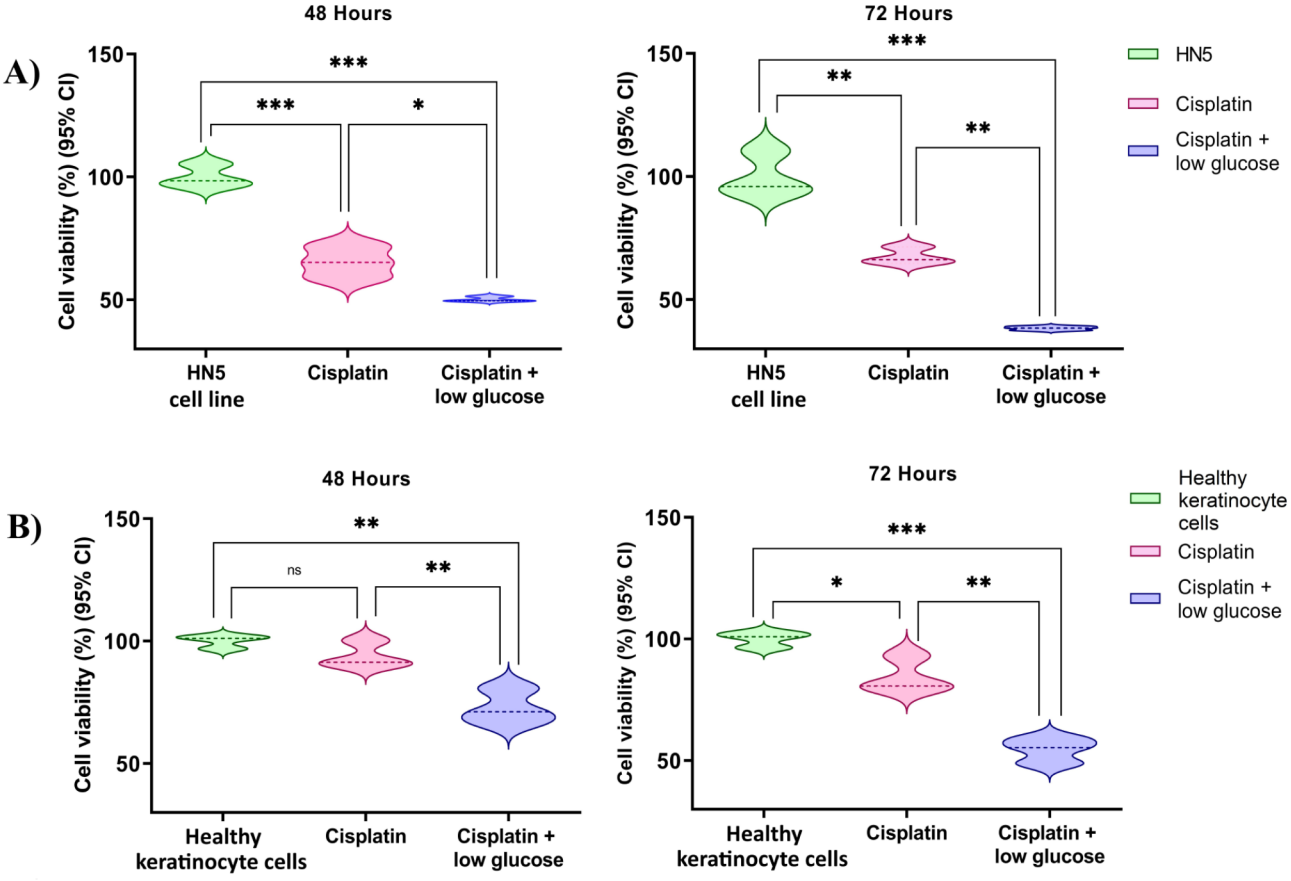

Fig. 1 MTT assay of cell viability: **A**) HN5 cell line after 48 and 72 h, **B**) healthy keratinocyte cells after 48 and 72 h. ns: p value > 0.05, *: p value ≤ 0.05, **: p value ≤ 0.01, ***: p value ≤ 0.001

was observed which not statistically significant ($p=0.421$) (Table 2; Fig. 1-B).

Cell apoptosis in HN5 and healthy keratinocyte cells

According to the Annexin-V test in the HN5 cells, the highest cell necrosis average was in the cisplatin group (SCC-2), the highest average of late apoptosis and early apoptosis, and the lowest average of live cells was related to cisplatin with low glucose groups (SCC-3). Relatively similar results were obtained in healthy keratinocyte cells. The highest mean of cell necrosis, late apoptosis, and early apoptosis and the lowest average of live cells were observed in the cisplatin with low glucose groups (Healthy-3) (Tables 3 and 4). The results of the one-way ANOVA analysis showed a statistically significant difference in the percentage of cell necrosis ($p=0.024$, $F=7.452$), late apoptosis ($p<0.001$, $F=82.506$), early apoptosis ($p=0.001$, $F=34.231$) and live ($p<0.001$, $F=254.450$) cells between the groups of HN5 cells, and significant difference in the percentage of cell necrosis ($p=0.182$, $F=2.297$), late apoptosis ($p=0.033$, $F=6.307$), early apoptosis ($p=0.274$, $F=1.619$) and live ($p=0.009$, $F=11.225$) cells between the groups of healthy keratinocytes ($p<0.05$). These results were found in both 48 and 72 h MTT assay (Table 3; Fig. 2).

Tukey's HSD analysis of HN5 cells revealed a statistically significant increase in both late and early apoptosis ($p<0.05$) in the cisplatin with low glucose group (SCC-3) compared to other groups (Tables 3 and 4; Fig. 2). Additionally, a significant decrease in live cells was observed in the cisplatin with low glucose group (SCC-3) ($p<0.05$). In contrast, cell necrosis was statistically higher only in the cisplatin group (SCC-2) compared to the control group, with no significant difference observed in the cisplatin with low glucose group (SCC-3). These findings were consistent at both 48 and 72 h as measured by the MTT assay.

For healthy keratinocyte cells, the cisplatin with low glucose group (Healthy-3) displayed a significant increase in necrosis, late apoptosis, and early apoptosis compared to other groups ($p<0.05$) (Table 4; Fig. 3). This was accompanied by a significant decrease in the number of live cells.

Discussion

For the first time, this study investigated the effect of STF on the HNSCC cell line. Based on the results of the study, short-term fasting for 48 h before chemotherapy significantly decreases the cell viability and increases the apoptosis of HNSCC cells compared to the control

Table 3 Tukey HSD for apoptosis of HN5 cell line

Cells and sequence of treatment			Mean difference	Standard error	*p* value	95% confidence interval for mean	
						Lower bound	Upper bound
Necrosis	HN5	Cisplatin	-5.246	1.386	0.021 *	-9.498	-0.993
		Cisplatin + low glucose	-1.712	1.386	0.477	-5.964	2.540
	Cisplatin	HN5	5.246	1.386	0.021 *	0.993	9.498
		Cisplatin + low glucose	3.533	1.386	0.096	-0.719	7.786
	Cisplatin + low glucose	HN5	1.712	1.386	0.477	-2.540	5.964
		Cisplatin	-3.533	1.386	0.096	-7.785	0.719
Late apoptosis	HN5	Cisplatin	-13.000	1.409	0.000 *	-17.322	-8.678
		Cisplatin + low glucose	-17.400	1.409	0.000 *	-21.722	-13.078
	Cisplatin	HN5	13.000	1.409	0.000 *	8.678	17.322
		Cisplatin + low glucose	-4.400	1.409	0.047 *	-8.722	-0.078
	Cisplatin + low glucose	HN5	17.400	1.409	0.000 *	13.078	21.722
		Cisplatin	4.400	1.409	0.047 *	0.0780	8.722
Early apoptosis	HN5	Cisplatin	-18.587	3.883	0.007 *	-30.500	-6.673
		Cisplatin + low glucose	-31.987	3.883	0.000 *	-43.900	-20.073
	Cisplatin	HN5	18.587	3.883	0.007 *	6.6734	30.500
		Cisplatin + low glucose	-13.400	3.883	0.031 *	-25.313	-1.487
	Cisplatin + low glucose	HN5	31.987	3.883	0.000 *	20.073	43.900
		Cisplatin	13.400	3.883	0.031 *	1.487	25.313
Live	HN5	Cisplatin	36.833	2.339	0.000 *	29.657	44.009
		Cisplatin + low glucose	51.133	2.339	0.000 *	43.957	58.309
	Cisplatin	HN5	-36.833	2.339	0.000 *	-44.009	-29.657
		Cisplatin + low glucose	14.300	2.339	0.002 *	7.124	21.476
	Cisplatin + low glucose	HN5	-51.133	2.339	0.000 *	-58.309	-43.957
		Cisplatin	-14.300	2.339	0.002	-21.476	-7.124

*: p value ≤ 0.05

Table 4 Tukey HSD for apoptosis of healthy keratinocyte

	Cells and sequence of treatment		Mean difference	Standard error	*p* value	95% confidence interval for mean	
						Lower bound	Upper bound
Necrosis	healthy	Cisplatin	-4.577	2.857	0.315	-13.344	4.191
		Cisplatin + low glucose	-5.813	2.857	0.185	-14.581	2.954
	Cisplatin	healthy	4.577	2.857	0.315	-4.191	13.344
		Cisplatin + low glucose	-1.237	2.857	0.904	-10.004	7.531
	Cisplatin + low glucose	healthy	5.813	2.857	0.185	-2.954	14.581
		Cisplatin	1.237	2.857	0.904	-7.531	10.004
Late apoptosis	healthy	Cisplatin	-7.431	3.754	0.198	-18.948	4.086
		Cisplatin + low glucose	-13.301	3.754	0.028 *	-24.818	-1.784
	Cisplatin	healthy	7.431	3.754	0.198	-4.086	18.948
		Cisplatin + low glucose	-5.870	3.754	0.330	-17.387	5.647
	Cisplatin + low glucose	healthy	13.301	3.754	0.028 *	1.784	24.818
		Cisplatin	5.870	3.754	0.330	-5.647	17.387
Early apoptosis	healthy	Cisplatin	-1.887	2.327	0.711	-9.026	5.253
		Cisplatin + low glucose	-4.180	2.327	0.249	-11.319	2.959
	Cisplatin	healthy	1.887	2.327	0.711	-5.253	9.026
		Cisplatin + low glucose	-2.293	2.327	0.612	-9.433	4.846
	Cisplatin + low glucose	healthy	4.180	2.327	0.249	-2.959	11.319
		Cisplatin	2.293	2.327	0.612	-4.846	9.433
live	healthy	Cisplatin	13.80	4.939	0.070	-1.354	28.954
		Cisplatin + low glucose	23.267	4.939	0.008 *	8.113	38.420
	Cisplatin	healthy	-13.80	4.939	0.070	-28.954	1.354
		Cisplatin + low glucose	9.467	4.939	0.214	-5.687	24.620
	Cisplatin + low glucose	healthy	-23.267	4.939	0.008 *	-38.420	-8.113
		Cisplatin	-9.467	4.939	0.214	-24.620	5.687

*: p value ≤ 0.05

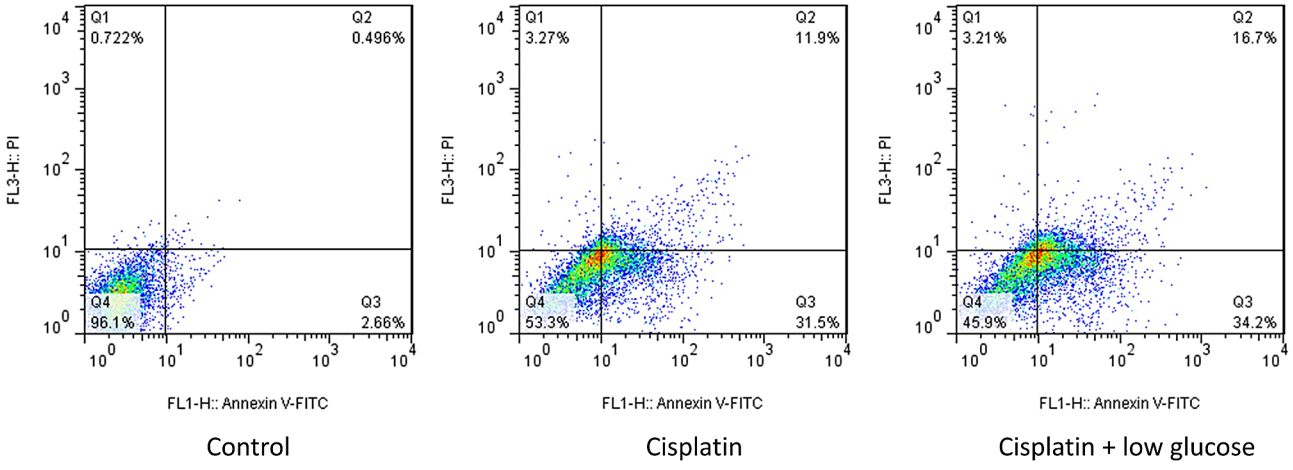

Fig. 2 Apoptosis results of HN5 cell line in in various group of treatment

group. However, the protective effect of this adjuvant method was not seen in preserving healthy keratinocyte cells against chemotherapy. Warburg's effect refers to the chemoresistance of the tumor, associated with high aerobic glycolysis and low oxidative phosphorylation [58]. Tumor-starved cells promote an anti-Warburg effect through increase in the translation of selected genes such as the AKT/S6K signaling pathway, which leads to an increase in cell respiration and oxygen consumption. This causes increased ROS, DNA damage, Caspase 3 activation, and apoptosis, especially during chemotherapy [10, 16, 59].

Therefore, declines in plasma levels of insulin-like growth factor-1 (IGF-1), insulin, and glucose mediate the effects of fasting on cancer cells by improvement of apoptosis. Since the reduction of glucose and IGF1 are

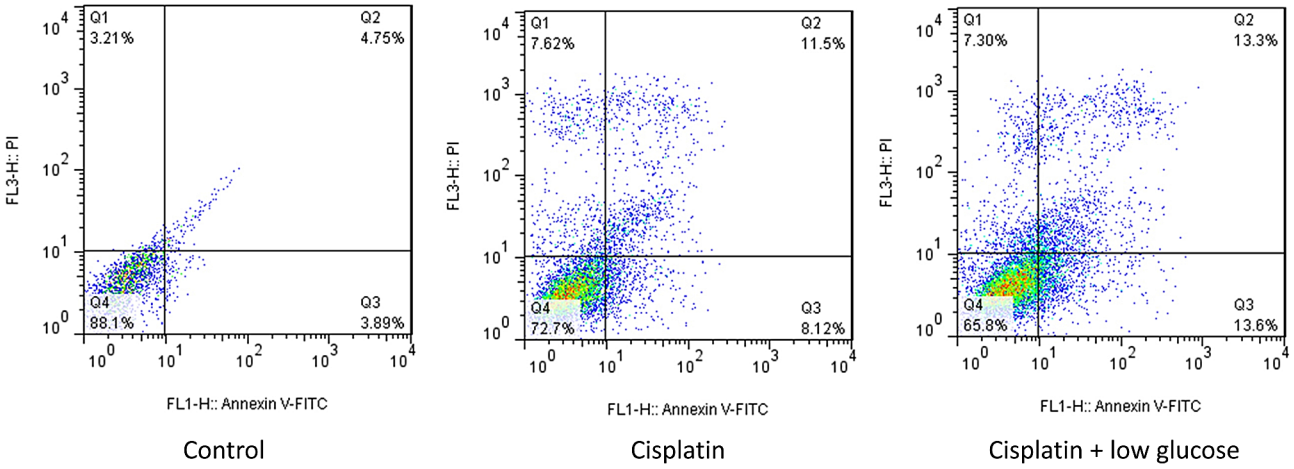

Fig. 3 Apoptosis results of healthy Keratinocyte cells in various group of treatment

the main mediators in the effectiveness of ketogenic therapy on cancer and healthy cells, the STF diet seems to be preferable to caloric restriction (CR) or intermittent fasting (IF) [9, 28]. Two studies showed that IF with or without CR did not significantly improve survival and reduce tumor growth in mice with prostate cancer [36, 37]. It is noteworthy that the decrease in blood glucose level after STF (2–5 days) is 75%, and the decrease in IGF-1 was 75%; however, after long CR or IF, only a 15% decrease in blood glucose and 25–30% decrease in IGF-1 have been reported [9].

Several previous studies confirmed the beneficial effects of short-term fasting during chemotherapy and radiotherapy [10, 16, 28]. Fasting adjuvant to chemotherapy drugs causes a significant reduction in tumor size, tumor weight, tumor bioluminescence, adenosine levels in the micro tumor environment, and upregulation of autophagy, which can prevent tumor progression and improve anti-tumor immunity in various cancer cell lines and higher survival rate in animal mode. Consistent with our findings, Lee et al. indicated higher apoptosis in glioma cells and 4T1 breast cancer cells following 48 and 72 h of STF before chemotherapy, respectively. However, in contrast to our result, he showed decreased apoptosis in healthy glia cells [10, 28]. Bianchi et al. also established that 48 h of STF can promote apoptosis in colon tumor cell lines [16].

In addition to laboratory and animal studies, some human studies have also shown the positive effects of fasting therapy in cancer treatment. De Groot et al. showed that a fasting-mimicking diet for four days before chemotherapy led to better therapeutic effects and radiological evidence than the control group [16]. According to the DSRP theory, fasting can improve the survival and preservation of healthy cells against the toxic effects of chemotherapy drugs. This effect was not confirmed on healthy keratinocyte cells in the current study. De Groot et al., in a pre-clinical study, stated that STF (24 h before and 24 h after) significantly protected from the hematological toxicity associated with chemotherapy. However, non-hematological toxicity, including fatigue, infection, mucositis, neuropathy, diarrhea, dizziness, nausea, constipation, and eye problems, did not differ between the two groups. They suggested that the reduction of hematotoxicity can be related to the lower intensity of bone marrow suppression or the reduction of the breakdown of circulating blood cells and possibly the faster recovery of DNA damage in peripheral blood mononuclear cells after chemotherapy in the Fasting group [60]. In other human studies, with increasing STF duration, especially before chemotherapy (48, 36, and 72 h), we see a reduction in hematological and non-hematological toxicity and an improvement in quality of life in treated patients [61–63]. Reducing the non-hematologic toxicity of chemotherapy compared to hematologic toxicity requires a relatively long fasting period, especially before the chemotherapy, and subsequently, a more significant reduction in the level of IGF-1. The reduction of chemotherapy side effects by STF has been reported even in studies that did not significantly improve the antitumor effects of chemotherapy. The strong reduction of active metabolites of chemotherapy drugs in the serum and liver of fasting group mice, despite the lack of difference in tumoral tissue [64], can justify the lower side effects and, simultaneously, similar therapeutic effects in the Fasting group.

Building on the findings of this study and aligning with the majority of previous research, STF appears to significantly enhance cancer treatment. Even in studies without statistically significant differences in treatment outcomes, STF did not impede chemotherapy's ability to reduce tumor size or markers. Furthermore, the potential reduction of chemotherapy side effects strengthens the case for STF as an adjuvant therapy. Importantly, all human and animal studies report good tolerability and

safety with the STF diet. Therefore, we propose further large-scale clinical trials investigating the effects of different STF durations and adjuvant chemotherapy drugs on tumor toxicity, along with hematological and non-hematological side effects. This research should encompass a diverse patient population representing various stages of HNSCC.

Conclusion

The finding of this study revealed that short-term fasting (48 h before and 48 h after) chemotherapy significantly improved HNSCC cell line apoptosis and necrosis; however, the protective effect of fasting therapy on healthy oral keratinocytes has not been established. This study lays the groundwork for future research avenues exploring the potential of Short-Term Fasting (STF) as a therapeutic strategy for HNSCC. Future investigations should delve deeper into the mechanism of action by employing Western blot analysis to pinpoint changes in apoptosis and cell cycle proteins. Additionally, studies with extended durations and varying fasting periods are warranted to assess long-term effects and dose-response relationships. Furthermore, exploring the potential of combining STF with chemotherapy drugs for synergistic anti-cancer effects or reduced toxicity on healthy cells holds promise. Finally, investigating the impact of STF on cell migration and invasion, as well as cytokine and inflammatory marker profiles, could provide valuable insights into its influence on the tumor microenvironment and its potential immunomodulatory properties. These future directions offer exciting possibilities for harnessing the power of STF in the fight against HNSCC.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions

N. Sh: Substantial contribution to the conception and design, drafting of the manuscript, critically revising the manuscript for important intellectual content, approval of the final version submitted for publication. "Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved."M. K: Substantial contribution to the analysis and interpretation of data, drafting the manuscript, and approval of the final version submitted for publication. "Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved."A H. A T: Substantial contribution to the acquisition and analysis of data, critically revising the manuscript for important intellectual content, and approval of the final version submitted for publication. "Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved."

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Data availability

The data supporting the findings of this study are available upon reasonable request from the authors.

Declarations

Ethics approval and consent to participate

The protocol of this study was approved by the ethics committee of Tehran University of Medical Science (IR.TUMS.DENTISTRY.REC.1400.124) and informed consent was obtained from all individuals.

Consent to publish

Not applicable.

Competing interests

The authors declare no competing interests.

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