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An Iodine Treatments Effect on Cell Proliferation Rates of Breast Cancer Cell Lines; *In Vitro* Study

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Abstract

BACKGROUND: Iodine can reduce breast tumor progression by mediates an antiproliferative effect.

AIM: This study aimed to investigate the effect of iodine (I2), Lugol (I3K), and the combination of both on cell proliferation of three different types of breast cancer cell lines.

METHODS: The samples were MCF7, SKBR3, and MDA-MB 213 cell lines. Cell proliferation rate was measured using colorimetric and clonogenic assays.

RESULTS: The cell proliferation rate of MDA-MB 231 cells was reduced significantly by treatment I_2, I_3K, and combination of both with $p = 0.046$, $p = 0.00$, and $p = 0.00$, respectively. In MCF7 cells, I_2 reduced the cell proliferation of 54–94% and I_3K reduced the proliferation of 74–94%. The effectiveness of I_3K treatments in slowing cell proliferation rate was dose-dependent. In SKBR3 cells, I_2 reduced proliferation cell up to 85% and I_3K 4%-94% depending on the dose. Clonogenic assay results showed a discontinue of the cell proliferation by all doses of I_2 and I_3K (10 μM and 20 μM).

CONCLUSION: Breast cancer cell lines, representing subtypes of luminal A, HER2+, and triple-negative, show an excellent response to iodine treatments and I_3K response shows in a dose-dependent manner. Further studies are needed to investigate the effective *in vivo* doses.

Introduction

Iodine plays a pivotal role in maintaining the mammary gland's normal healthy integrity and its deficiency transmutes a healthy structure and function of rat mammary gland [1], [2]. Some of the studies reported that iodine mediates apoptosis and has an effect on antineoplastic process [3], [4], [5]. Iodine consumption of 3–6 mg/day has reduced breast hyperplasia, per lobular/ductal fibrosis, and slowing down breast cancer progression [6], [7]. Iodine mediates antiproliferative effects directly or indirectly. Oxidized iodine eliminates the mitochondrial membrane potential (MMP) to mediate apoptosis directly or by forming iodolipids and activation of gamma type peroxisome proliferator-activated receptors γ (PPARγ) that trigger apoptosis [2], [5], [8], [9].

Cancer disease is the second leading cause of death globally; it caused more than ten million death annually [10], [11], [12]. The incidence rate of breast cancer in the United States (US) increased slightly by 0.3% per year, but the mortality rate declined by around 40% from 1989 to 2017 [13]. The Asian region has the lowest breast cancer mortality in the world [5]. Globocan report 2018 said that new breast cancer cases in the US are higher than in Japan (7.5%:11%) [12]. Seaweed is a common dietary component in Japanese and it is a rich source of iodine. Japanese iodine consumption is 25 times higher than in Western countries. It has been associated with the low incidence of breast cancer in Japanese people compared to Western [2]. However, breast cancer number in Japan continues to increase that may be exposed to the western diet and lifestyle [14].

Breast cancer was classified based on histopathology findings. Advanced techniques such as immunohistochemistry define the molecular subtypes based on the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 positive (ERBB2/HER2+) [15], [16], [17]. Identification of the molecular subtypes of breast cancer is essential for a personalized treatment plan and predict prognosis. Some breast cancer subtypes are resistant to some therapies [15], [17], [18]. Limitation of the benefit of available therapy challenges an emerge of new alternative therapy for breast cancer.

The total amount of iodine in the human body is around 30–50 mg. Less than 30% presents in the thyroid gland. Around 60–80% is concentrated in extrathyroidal tissues. These include salivary glands, gastric mucosa, lactating mammary gland, the choroid plexus, and et cetera [1], [19]. Non-lactating breast tissue is known to be NIS and peroxidase poor and does not provide for organification [4].

I_2 is taken up by a non-lactating mammary gland without the involvement of NIS and peroxidase activity, and it is a highly reactive species [2], [4], [20]. I_2 mediates antiproliferative effect with direct or indirect action. *In vitro* studies showed that I_2 exposure induced the mitochondria to release apoptosis factors that cause nuclear fragmentation directly [4], [21]. On the other hand, breast tumor tissues contain arachidonic acid (AA) more than the normal ones. Iodination of AA formed 6-iodolactone (6-IL), which is an essential membrane lipid. PPARγ could be the mediator of the 6-IL apoptotic effect [1], [3], [5].

This study investigates the proliferation rates of I_2, I_3K, or a combination of both in various concentrations treatments of three types of breast cancer cell lines. It will obtain the potential extrathyroidal benefits of iodine and alternative breast cancer therapy.

Materials and Methods

Cell lines and culturing

This study uses three types of breast cancer cell lines MCF7 (estrogen and progesterone positive receptors/ER+ and PR+) and SKBR3 (human epidermal growth factor receptor 2+/HER2+) and MDA-MB 231 (triple-negative) [18]. SKBR3 was purchased from the American Type Culture Collection, MCF7, and MDA-MB 231 cell lines were gifted from the Faculty of Medicine, Universitas Padjadjaran, Bandung-Indonesia. MCF7 and MDA-MB 231 cells were cultured in RPMI 1640 medium (Invitrogen). SKBR3 was cultured in McCoy's 5A medium (Invitrogen). Mediums were added with 10% fetal bovine serum (Invitrogen), 1% penicillin, 1% streptomycin, and 1% amphotericin B. The cells were incubated at 37°C and supplied with 5% carbon dioxide (CO_2) until 90% confluence was reached.

Proliferation rates

The cells' proliferation rate was measured using a colorimetric assay and clonogenic test.

Colorimetric assay

Methyl thiazolyldiphenyl-tetrazolium bromide for iodine and Lugol and radioiodine treatments was used to measured cell proliferation rate. The cultured cells were washed with phosphate-buffered saline (PBS) twice, then seeded in a 96-well plate at a density of ×2 10^4/well and incubated for 24 h then treated with 1 μM, 10 μM, 20 μM of I_2, I_3K, and a combination of I_2 and I_3K. The treatments were done for 24, 48, and 72 h, and it carried out in triplicate. The control cells were treated with medium only. MTT (5 mg/ml in PBS) was added to the wells, and the plate was incubated for 4 h at 37°C. The medium was removed, and 100 ml dimethyl sulfoxide was added to the wells, and the absorbance was measured at 550 nm using a colorimetric.

The proliferation rate was calculated by the equation:

$$\text{Cell proliferation}(\%) = \frac{\text{OD test} - \text{OD blank}}{\text{OD control} - \text{OD blank}} \times 100$$

Clonogenic assay

Cells were grown in six-well culture plates 100 cells/well, for a day. The next day, the cells were incubated for 72 h in 5% CO2 at 37°C with 1 μM, 10 μM, and 20 μM of I_2, I_3K, and a combination of both. The reaction was terminated by removing the iodine-containing medium, and the cells were washed twice with cold PBS. The cells were then grown for 12 days in the mediums. They were supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin, 1% streptomycin, and 1% amphotericin B. The cells were then washed with PBS, fixed with methanol/water (1:1), and then stained with crystal violet. Macroscopic colonies were counted. These experiments were performed twice. The proliferation rate was calculated as the number of cell colonies in plates treated with I_2, I_3K, and a combination of both compared with those untreated cells.

Statistical analysis

All experiments were performed in triplicate. Results are presented as means ± standard deviation. Statistical significance comparing experimental values used non-parametric tests (Kruskal–Wallis and Mann–Whitney test). One-way ANOVA and post-hoc-Bonferroni for the parametric test were performed.

Results

MDA-MB 231 cell lines

The averages of the cell proliferation after treatments of I_2, I_3K, and combination of I_2–I_3K (1 μM) were 98 ± 5%, 88 ± 33%, and 110 ± 3%, respectively, 95 ± 6%, 80 ± 6%, and 79 ± 7% respectively, and 55 ± 8%, 53 ± 8%, and 47 ± 7%, respectively, as shown in Table 1. The cell

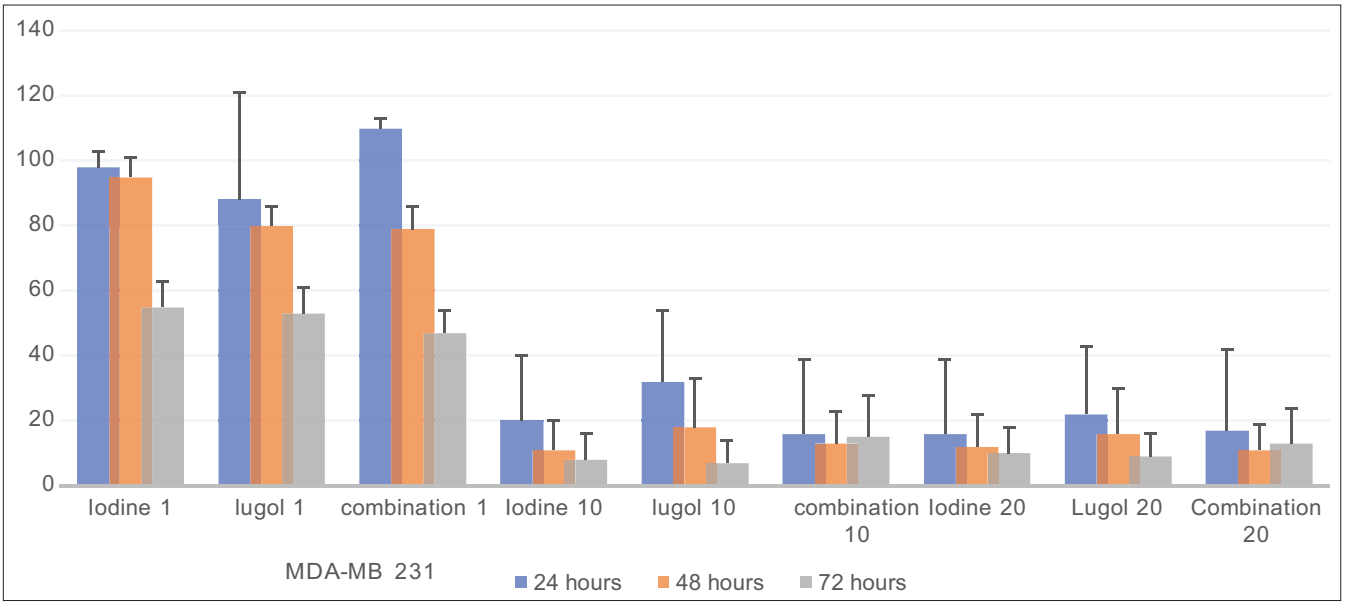

Figure 1: MDA-MD 231 cell proliferation rate of various treatments of iodine, Lugol, and combination of both for 24, 48, and 72 h. The proliferation rates reduced significantly with treatments 10 μM and 20μM compared to 1 μM (p = 0.001, p = 0.001, and p = 0.001), respectively.

Table 1: MDA-MB231 cells proliferation rate after 1, 10, and 20 μM of I_2, I_3K and a I_2-I_3K combination

MDA-MB 231 (%)	Cell proliferation Means ± SD (%) 24 h	Cell proliferation Means ± SD (%) 48 h	Cell proliferation Means ± SD (%) 72 h	p
I_2				
1	98 ± 5	95 ± 6	55 ± 8	0.001
10	20 ± 20	11 ± 9	8 ± 8	0.590
20	16 ± 23	12 ± 10	10 ± 10	0.940
p	0.061	0.055	0.063	
I_3K				
1	88 ± 33	80 ± 6	53 ± 8	0.170
10	32 ± 22	18 ± 15	7 ± 7	0.360
20	22 ± 21	16 ± 14	9 ± 7	0.730
p	0.079	0.061	0.055	
I_2 + I_3K				
1	110 ± 3	79 ± 7	47 ± 7	0.490
10	16 ± 23	13 ± 10	15 ± 13	0.449
20	17 ± 25	11 ± 18	13 ± 16	0.709
p	0.066	0.055	0.066	

Table 2: MCF7 cells line proliferation rate after 1, 10, and 20 μM of I_2, I_3K and a I_2-I_3K combination

MCF7 (%)	Cell proliferation Means ± SD (%) 24 h	Cell proliferation Means ± SD (%) 48 h	Cell proliferation Means ± SD (%) 72 h	p
I_2				
1	60 ± 7	66 ± 31	27 ± 17	0.144
10	52 ± 6	16 ± 11	17± 9	0.010
20	28 ± 21	25 ± 19	6 ± 4	0.280
p	0.113	0.099	0.177	
I_3K				
1	140 ± 56	114 ± 98	162 ± 22	0.689
10	120 ± 34	30 ± 16	26 ± 10	0.001
20	36 ± 24	6 ± 6	12 ± 10	0.154
p	0.055	0.042	0.001	
I_2+I_3K				
1	273 ± 106	103 ± 13	105 ± 11	0.021
10	115 ± 28	33 ± 9	21 ± 9	0.065
20	32 ± 22	14 ± 6	14 ± 11	0.275
p	0.010	0.000	0.000	

proliferation rate of MDA-MB 231 cells was reduced significantly by treatment I_2, I_3K, and combination of both with p = 0.040, p = 0.001, and p = 0.001, respectively, as shown in Figure 1. Treatment doses of 10 μM and 20 μM reduced cell proliferation significantly compared to the dose of 1 μM with p = 0.001 and p = 0.001.

The antiproliferation rate of I_2, I_3K, and combination of both for 24, 48, and 72 h treatment, not differ significantly. The treatment combination did not show different results compared to I_2, I_3K alone p = 0.177, p = 0.066, and p = 0.329.

MCF7 cell lines

The averages of the cell proliferation after treatments of I_2, I_3K, and combination of I_2, and I_3K (1 mM) for 24 h were 60 ± 7%, 140 ± 56%, and 273 ± 106%, respectively, for 48 h were 66 ± 31%, 114 ± 98%, and 103 ± 13%, respectively, and for 72 h were 27 ± 17%, 162 ± 22%, and 105 ± 11%, respectively, as showed in Table 2. Iodine treatment showed a reduced cell proliferation rate of MCF7, and the effect did not make a significant difference between those doses, as shown in Figure 2. Treatment of Lugol 10 μM and 20 μM reduced cell proliferation significantly compared to Lugol 1 μM, with p = 0.001 and p = 0.001, respectively. The combination of I_2 and I_3K 10 μM and 20 μM reduced cell proliferation significantly compare to 1 μM dose, with p = 0.001 and p = 0.010, respectively. There is no significantly different effect on cell proliferation between combination treatments of I_2 and I_3K of 10 μM with 20 μM.

SKBR3 cell lines

The averages of the cell proliferation after treatments of I_2, I_3K, and combination of I_2 and I_3K (1 μM) for 24 h were 29 ± 10%, 96 ± 20%, and 93 ± 23%, respectively, for 48 h were 25 ± 21%, 141 ± 75%, and 111 ± 70%, respectively, and for 72 h were 27 ± 17%, 335 ± 216%, 242 ± 80%, respectively, as shown in Table 3. Iodine reduced proliferation cell up to 85%. The antiproliferative effect did not differ between I_2 treatment doses, as shown in Figure 3. However, the

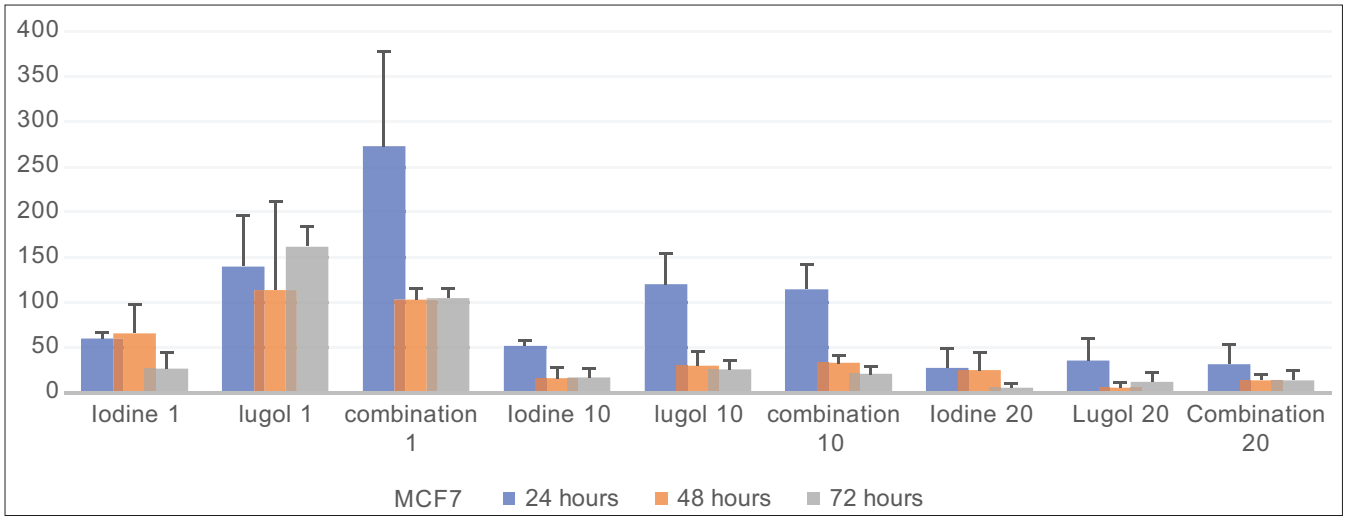

Figure 2: MCF7 cell proliferation rate of various iodine, Lugol treatments, and a combination of both for 24, 48, and 72 h. Iodine treatment doses reduced cell proliferation did not depend on the dose. Lugol and a combination of I_2 and I_3K (10 μM) showed an antiproliferative effect after 48 and 72 h of treatments p = 0.010 and p = 0.001, respectively

Table 3: SKBR3 cells proliferation rate after 1, 10, and 20 μM of I_2, I_3K and combination of I_2-I_3K treatments

SKBR3 (%)	Cell proliferation Means ± SD (%) 24 h	Cell proliferation Means ± SD (%) 48 h	Cell proliferation Means ± SD (%) 72 h	p
I_2				
1	29 ± 10	25 ± 21	27 ± 17	0.220
10	15 ± 3	17 ± 15	15 ± 10	0.988
20	16 ± 16	19 ± 14	24 ± 15	0.226
p	0.236	0.381	0.413	
I_3K				
1	96 ± 20	141 ± 75	335 ± 216	0.148
10	16 ± 6	15 ± 10	11 ± 10	0.560
20	6 ± 6	24 ± 18	10 ± 6	0.582
p	0.001	0.069	0.006	
I_2 + I_3K				
1	93 ± 23	111 ± 70	242 ± 80	0.23
10	11 ± 8	17 ± 12	16 ± 14	0.94
20	14 ± 12	21 ± 10	25 ± 11	0.83
p	0.001	0.215	0.008	

Table 4: The clonogenic assay results in MDA-MB 231, SKBR3, and MCF7 cell lines after 1 μM, 10 μM, and 20 μM of I_2, I_3K treatments

Doses (%)	MDA-MB 231	SKBR3	MCF7
I_2			
1	0	0	0
10	0	0	0
20	0	0	0
I_3K			
1	230 ± 25 (62.8%)	208 ± 34 (39.8%)	284 ± 51 (71%)
10	0	0	0
20	0	0	0
Control	366 ± 8.5	523 ± 28.3	400 ± 28.3

treatment effect of I_3K and a combination of I_2 and I_3K depends on the dose, with p = 0.001 and p = 0.001 for 24 h, respectively, and p = 0.006 and p = 0.008 for 72 h, respectively. The combination of I_2 and I_3K (1 μM) had no different results with I_3K (1 μM) alone, with p = 0.623.

Clonogenic result

Iodine treatment in varying doses discontinues the cell proliferation, as shown in Table 4 and Figure 4. Even though I_3K 1 μM dose did not affect in the slowing of cell proliferation, but 10 μM and 20 μM doses suppressed cell proliferation.

Discussion

In thyroid cell and lactating mammary gland, iodide transport is facilitated by NIS [6], [22]. Iodide excess induces apoptosis in thyrocytes, and the effect depends on thyroid peroxidase [3]. On the other hand, non-lactating mammary gland has a low level of peroxidase. The condition does not provide the environment for iodide organification. However, I_2 can be utilized without sodium iodide symporter and peroxidase [19], [23]. I_2 treatment in benign breast cancer has been found to reduce tumor size and symptoms, but not I^- treatment [6]. Iodide is preferentially taken up and retained in the thyroid and lactating mammary glands, but the pituitary, ovary, and non-lactating breast glands, I_2 are taken up more readily than I^- [6], [22].

Iodine in breast tissue was explained by *in vitro* studies that I_2 is taken up through diffusion, then incorporated into iodolipids and nuclear components, then exert antiproliferative and apoptotic properties [3], [19], [24]. Previous studies have reported that the antiproliferative effects of I_2 are through MMP destruction that directly results in apoptosis. MMP is needed for mitochondrial functions such as ATP production, metabolite transport regulation, and protein import. The mitochondrial intermembrane space contains several proteins that induce apoptosis through cytochrome c induction or activation of a caspase-independent apoptotic death program [3], [8], [9], [25]. Furthermore, iodine's antiproliferative effect can occur through iodolipid formation, 6-IL, or alpha-iodohexane canal, and the activation of PPARγ interaction through increased BAX, caspases 3, 7, p53, and p21 [1], [3], [21], [25].

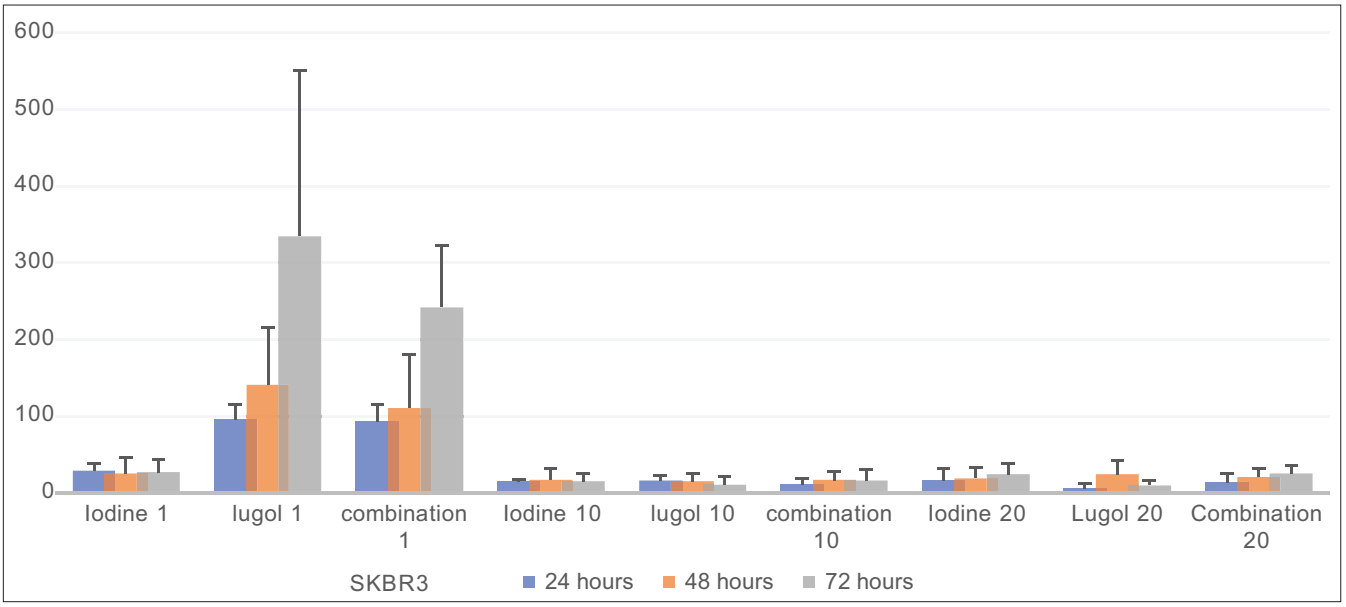

Figure 3: SKBR3 cell proliferation rate of the various treatment doses of iodine, Lugol, and a I_2-I_3K combination for 24, 48, and 72 h. Iodine reduced the cell proliferation did not depend on the dose. Lugol and combination iodine and Lugol showed reduced cell proliferation with 10 μM and 20 μM with p = 0.001 and p = 0.001, respectively

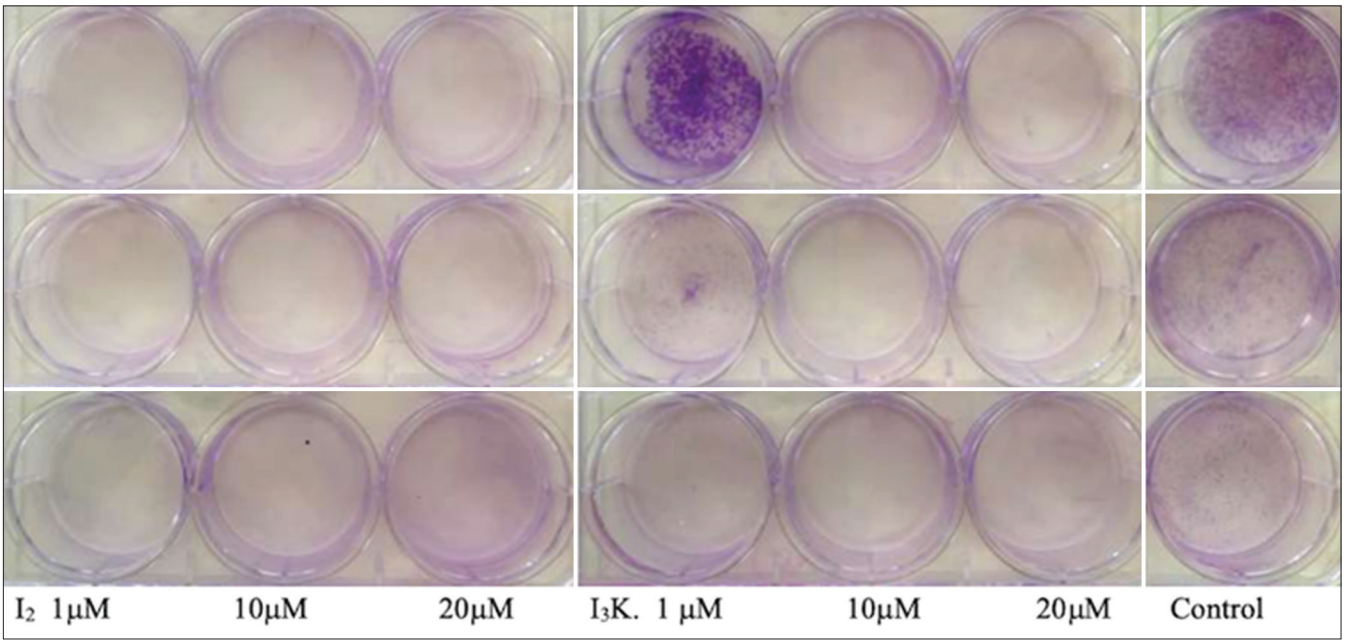

Figure 4: Clonogenic assay results, as shown on MCF7 (top), MDA-MB231 (middle), and SKBR3 (below). From left to right are results of the clonogenic assay of 1, 10, and 20μM of I_2 and I_3K and control cells. I_2 suppresses cell proliferation in all doses, and the same effect showed in 10 μM and 20 μM of I_3K

Recently, a study has reported that I_2 treatment did not have an antiproliferative effect in MDA-MB 231 cells [4]. This study found that the proliferation MDA-MB 231 cell discontinued by I_2 treatment, as shown in Figures 1 and 4. The low dose of I_3K (1 μM) showed a limitation of the MDA-MB 231 cells' antiproliferation effect. However, 10 μM and 20 μM of I_3K and a combination of I_2 and I_3K reduced cell proliferation significantly with $p < 0.05$ (Table 1). A combination of treatments did not show an increasing effect when it is compared with I_2 and I_3K alone. I_3K reduces cell proliferation effect in this study, in line with a study by Rösner *et al*. [6]. The clonogenic result showed that I_2 doses inhibited cell proliferation and I_3K (10 μM and 20 μM), as shown in Figure 4. However, the mechanism of iodine induces apoptosis in MDA-MB 231 has yet to be elucidated.

Meanwhile, I_2 inhibited the proliferation of MCF7 cells was not a dose-dependent manner (Table 2). The treatment reduced cell proliferation by up to 94%. This finding has a similar result with previous studies [3], [5], [26]. Furthermore, a study

reported that I_2 treatment was found to inhibit MCF7 cell growth, that even more effective than in MDA-MB 231 cells [1], [21]. In this study, I_2 treatments show similar effective in MCF7, MDA-MB 231, and SKBR3 cells from the clonogenic result. I_2 treatment increases the apoptotic rate and PPARγ expression, leading to a decrease in proliferation and dissipating the estrogen receptor alpha's translocation to the nucleus [3]. As MCF7 and MDA-MB 231 cells express PPARγ receptors, it can be hypothesized that the antiproliferation mechanism of I_2 in both cell lines is likely to be through iodine-PPARγ receptor interactions [21], [27]. Further studies are needed to explore it. Furthermore, I_3K treatments showed an antiproliferation effect on MCF7 exerts a dose-dependent manner. Nevertheless, a combination of I_2 and I_3K inhibited cell proliferation did not increase significantly compared with I_3K treatment alone, as shown in Table 2. The clonogenic result showed that I_3K (10 μM and 20 μM) effectively to stop cell proliferation (Figure 4).

SKBR3 cells do express NIS and showed that they could take radioiodine [28], [29]. In this study, I_2 and I_3K showed an antiproliferative effect, as shown in Table 3. The iodine effect did not change by a different dose. However, antiproliferative effect of I_3K and the combination of I_2 and I_3K tend to increase with higher doses (Table 3). The clonogenic result showed I_3K (10 μM and 20 μM) effectively to discontinued SKBR3 cell proliferation (Table 4). Further investigations are needed to elaborate on the mechanism of the apoptotic process of iodine exposure in breast cancer.

Conclusion

Iodine treatment was sufficient to discontinue cell proliferation of MCF7, MDA-MB231, and SKBR3 cells. The antiproliferative effect of I_3K shows in a dose-dependent manner. Breast cancer cell lines, representing subtypes of luminal A, HER2+, and triple-negative, show an excellent response to iodine treatments. Further studies are needed to investigate the effective dose of iodine to breast cancer subtypes *in vivo* and the effect toward the normal cell. This finding opens the opportunity of using iodine for breast cancer prevention as well as an alternative therapy.

Acknowledgments

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Ethical clearance

The subject in this research was cell lines; the authors did not have any contact with human or animal subjects, hence, not applicable.

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