ESTIMATION THE EFFECTS OF PLASMA ACTIVATED MEDIUM ON SOME COMPONENTS THAT RELATED WITH GROWING BREAST CANCER CELL LINES

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ABSTRACT

This study was aimed to assess the influence of plasma activated medium (PAM) on some parameters of breast cancer cell lines (MCF7 and AMJ13) in comparison to normal adipose-derived stem cells (ASCs). First, nitric oxide (NO) production within the used media (RPMI-1640 and MEM) was examined beyond different exposure time (10,15,20, and 25) minutes directly, and the finding suggested a significant increase in NO with increasing exposure time particularly after 20 and 25 min of irradiation in RPMI, while MEM showed significant results only after 25 min. The metabolic parameters were studied including glucose uptake and lactate dehydrogenase (LDH) activity. The results found a decrease in LDH activity proportional to incubation times, as over incubation to 48 hrs the activity reduced more significantly than the 24 hours with minimal influence on the enzyme activity in the media. Correlated results were demonstrated in glucose results as it's concentration was elevated in the media with increasing irradiation doses for all three treated cell lines in compared to untreated homologues.

Keywords :Plasma activated medium, lactate dehydrohenase, glycolysis, , and nitric oxide

مجلة العلوم الزراعية العراقية- 2024:55(1):342-352 فاضل وآخرون

تقدير تأثيرات الوسط المنشط بالبلازما على بعض المكونات المرتبطة بنمو سلالات خلايا سرطان الثدي

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المستخلص

هدفت هذه الدراسة إلى تقييم تأثيرالوسط الزرعي المنشط بالبلازماعلى بعض العوامل لخطوط خلايا سرطا ن الثدي MCF7 و AMJ13 مقارنة بالخلايا االجذعية المشتقة من الدهون الطبيعية ASCs . أولاً،تم فحص إنتاج أكسيد النيتريك (NO) داخل الاوساط الزرعية المستخدمة RPMIو MEM ، واظهرت النتائج زيادة ملحوظة فيNO بعد استخدام اوقات تعرض مختلف (10و15و 20و 25)دقيقة مع زياد ة وقت التعريض خصوصاً بعد 20 و 25 دقيقة من التعريض في RPMI ، بينما أظهر MEM نتائج مهمة فقط بعد 25 دقيقة. اضافة الى ذلك تمت دراسة المتغيرات الأيضية بما في ذلك امتصاص الجلوكوز ونشاط انزيم اللاكتات ديهيدروجينيز(LDH) . وجدت النتائج انخفاضًا في نشاط الانزيم متناسبًا مع أوقات الحضن،حيث انخفض النشاط بعد فترة 48 ساعة من الحضن بشكل ملحوظ مع تأثير ضئيل على نشاط الإنزيم في الوسطين. كانت هذه النتائج ذات الصلة مع نتائج الجلوكوز حيث ارتفع تركيزه في الاوساط مع زيادة جرعات التعريض لجميع خطوط الخلايا الثلاثة المعاملة مقارنة بذات الخطوط الخلوية غيرالمعاملة.

الكلمات المفتاحية: الوسط المنشط بالبلازما,وكسيد النتريك, انزيم لاكتيت ديهيدروجينيز,التحلل السكري

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INTRODUCTION

Plasma is the fourth matter of state following solid, liquid, and gas. It's found in two forms; thermal and cold plasma. The non-thermal plasma is a promising arising technology in the medical field as it's an ionized gas near room temperature, with multiple active components, such as the electrons, ions, free radicals, reactive molecules, photons, and ultraviolet radiation (27, 8, 12). Cold plasma can be used directly and indirectly to stimulate physiological mediators and produce reactive species around these cells. Plasma activated solution can be generated by exposing the liquid medium to plasma gas and retaining some reactive species created by the interaction with ions and electrons. Besides, the liquids that can be used in plasma activated medium (PAM) are: water, biological culture media, and other liquid forms that are used for cancer research, making it a more flexible tool for cancer treatment (15, 13, 17).
Iraqi Cancer Registry survey indicated that breast cancer is the first cancer among women which affects about 32% of the Iraqi population and it was also mentioned that about one-third of the cancers in women is breast cancer. However, the survival percentage is better than other fatal cancers, as the breast tissues are not a vital organ for human survival (4,10).
Metabolic reprogramming is considered a hallmark in cancer, as it increases from tumor aggressively. Many cancer cells prefer the anaerobic glycolysis rather than mitochondrial oxidative phosphorylation even with the presence of oxygen (11), as the high rate of glycolysis is considered advantageous to highly proliferative cancer cells for many causes: high ATP enough for rapid tumor growth, production of intermediate like glucose 6-phosphate and pyruvic acid can be used for the anabolism of fatty acids and nucleic acids, and the conversion of pyruvate to lactate which acidifies the micro-environment and makes it inappropriate for the growth of normal cells and promote the invasion of cancer one (28). The conversion of pyruvate to lactate is catalyzed by the lactate dehydrogenase enzyme which is found in all human cells. In addition, its well-recognized marker of tissue damage and can be indicated in serum due to pathological conditions such as cancers or cell necrosis (11, 26). This study aimed to target the metabolic pathway of breast cancer through plasma-activated medium treatment.

MATERIALS AND METHODS

Plasma jet device

Plasma jet instrument was designed and supplemented from University of Baghdad/College of sciences/Department of physics. In brief tungsten electrode was inserted in quartz tube (16cm) in length with outer and inner diameters of 0.5 and 0.3cm. The distance between the tip of the electron and the opening nozzle is 1 cm and supplied with alternating current (AC) power supply with a voltage of (18-20Kv). Argon was the working gas and the feeding was controlled by flow meter which regulated the input of gas to 3 L/min flow rate. (5).

Cell lines cultivation

Table 1 shown the used cell lines and there culturing conditions

Preparation of plasma activated medium

Three ml of each type of media (RPMI-1640 and MEM) was placed in tissue culture plate of 3 cm in diameter (SPL life science, Korea) treated with plasma jet for different exposure times (5,10,15, and 20 minutes) at 0.5cm distance, left to get cold for 5 minutes at room temperature then 2 ml of PAM were added to the six well plates containing cell lines. (25).

Determination of pH

The hydrogen number was determined after applying both MEM and RPMI-1640 media to plasma jet irradiation at different time intervals (10,15,20, and 25 minutes) by using pH strips (Citotest scintfic.co., Ltd). The strip was emerged in the treated medium for one second and then removed. After 10-15 seconds the strip color were compared with the chart card with different color each one corresponds to specific pH value **(**21).

Table 1. The used cell lines and their culturing conditions

Cell line	Cultivation and incubation conditions	Origin
MCF7	This cell line is isolated from Caucasian women of age 69 years old in 1970. A concentration of 1×10^5 cell/ml was cultured in MEM culture medium (Biochrom, German) with the addition of fetal bovine serum 10% (FBS; Biochrom, Germany), 1%penicillin-streptomycin (TROGE, Germany). Incubated at 37°C with 5% CO2.(18)	All cell lines were provided by the Experimental therapy department / Iraqi Center for Cancer and Medical Genetics Research (ICCMGR)
AMJ13	This cell line was cultured in the Iraqi Center for Cancer Research and medical genetics from70 of age Iraqi female. were cultivated in RPMI-1640 (USA), at a concentration of 1×10^5 cell/ml, 10% FBS and 1% antibiotic (penicillin-streptomycin) were added. Cells were incubated at 37°C in 5% CO2 (4)	
ASCs (Adipose-derived stem cells)	This cell line was cultured in the the Iraqi Center for Cancer Research and medical genetics. It was cultivated in RPMI-1640 (USA), at a concentration of 1×10^5 cell/ml, 10% FBS and 1% antibiotic (penicillin-streptomycin) were added. Cells were incubated at 37°C in 5% CO2 (20).	

Nitric oxide concentration measurement

The concentration of nitric oxide in µmol/L within the cell culture media represented in (RPMI and MEM) were evaluated by NO colorimetric assay kit, following the Elabscince kit assay protocol. Briefly, the absorbance of plasma activated medium supernatant was calculated for the examined groups which were treated at different interval times (10, 15,20,and 25 min) at 550 nm by the ELISA plate reader and the concentration calculated according to the given equation: (9)

$$\mathbf{NOConcentration}\left(\mu\frac{\mathbf{mol}}{\mathbf{l}}\right) = \Delta\frac{\mathbf{A1}}{\mathbf{\Delta A2}}\times\mathbf{C}\times\mathbf{f}$$

Where:

ΔA1=OD for sample-OD for Blank

ΔA2= OD for standard-OD for Blank

C= concentration of standard (sodium nitrate)

F=Dilution factor of sample

Glucose uptake analysis

Glucose was enzymatically measured in the media of cell culture treated with cold atmospheric plasma jet at different intervals time (10,15,20 min, and 25 min). Glucose concentration was measured by cobas integra Glucose HK Gen. 3 system (Glucose HK Gen 3; Roche Diagnostics, Indianapolis, IN) which use a cassette contains an in-vitro diagnostic reagents specific for this system device (Roche Medical Kit .Indianapolis; Roche Diagnostics) for the quantitative measurement of glucose in biological fluids. The measurement range is 0-40 mmol/L (0-720 mg/dL) (6)

LDH enzyme activity detection

Dependent on the results concluded above, LDH activity was measured after suitable timeof treatment with cold plasma jet device and it was detected in both supernatant of culture media and supernatant of cell lysate via the cobas integra Lactate Dehydrogenase acc. IFCC ver.2 reagent system (Roche Diagnostics, Indianapolis, IN) which identify the activity of lactate dehydrogenase enzyme by measuring the absorbance of cell lysate and media supernatant at a wave length of 340nm. The detection range falls between (10-1000U/ml) (3).

Statistical analysis

All data in this paper was analysis by graph pad prism software version 9, student t test and two way ANOVAs were used for the analysis .The data expressed as mean ±standard deviation (SD) of at least duplicate (5).

RESULTS AND DISCUSSION

Plasma jet impact on the pH of media

Treating the media with argon plasma jet didn't influence the pH of both RPMI-1640 and MEM which was stable at 7.4. In the same context, the temperature remained below 30 ºC which was measured by scanner thermometer gun (Etekcity/ USA), The temperature was

above the room temperature at about 2ºC. Together, these data suggest that the buffering capacity of the media is capable to maintain neutral pH in the range 7.2-7.4, which is suitable for the growth of the cells. As well as, addition of sodium bicarbonate to the synthetic media is increases the buffering and to counteract the CO2 effect and reduce the acidic environment resulting from metabolism (28).Prior results confirm that despite the reactive species generation, argon plasma didn't confer any change in the pH of treated RPMI 1640 (14). Furthermore, a Portugalian study indicated that medium irradiation did not result in a significant alteration in pH value which is in the range of 7.45 ± 0.02 at 20 ° C or in the temperature, which remained beneath 30°C (2).

Nitric oxide measurement in culture media prior to cell line culturing: The result of nitric oxide (NO) in RPMI was increased proportionally to increasing exposure time from 10 to 25 minutes. Results were (117.9±7.85, 129.4±4.35µmol/l) for 10 and 15 min. respectively, with non-significant difference was recorded. Whereas, the results of 20 and 25 min were significantly being differ as (187.5±12.5;p=0.047 µmol/l) and (253.7±8.3; p=0.048 µmol/l) respectively. While, different results were shown in MEM medium as the 10,15, and 20 min data were non-significant (88.5±3.5,142±22, 170±0.5 µmol/l) respectively. Unlike the 25 min which accompanied with significant differences (220±6;p=0.014 µmol/l). Thus, it was suggested that the other reactive species will also be increased linearly. Regarding the concentration of NO in different media, the data of the RPMI medium shows an increase in nitric oxide concentration than MEM medium as shown in table 2 and figure 1. This may be due to the fact that the chemistry of these media is very sophisticated as there are about thirty different compositions in both media including amino acids, serum type (fetal or calf), salt, pyruvate, and another component that contribute to the cytotoxic effect.Consequently, the increase in the organic compound in the media will elevate the cytotoxicity of PAM against breast cancer cell lines. For instance, Kim and Kim,(16) mentioned that anticancer activity differs within the same type of media depending on amino acids composition.

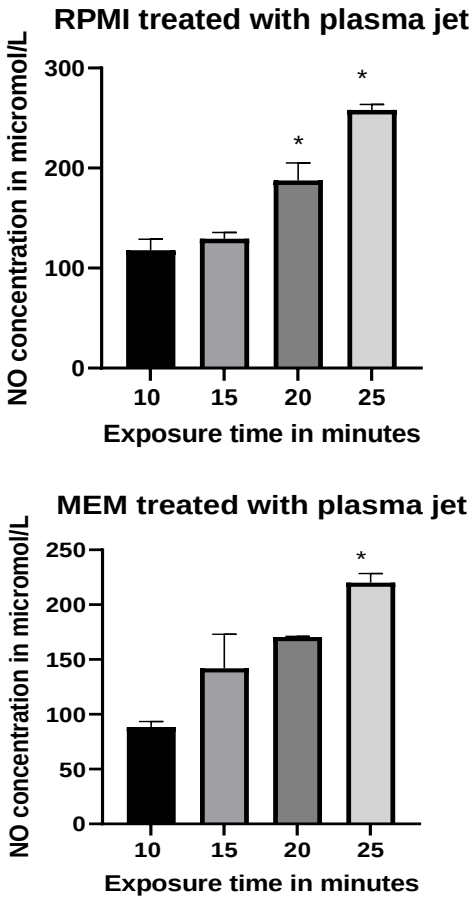

Figure 1. Nitric oxide concentration at different exposure time in RPMI-1640 and MEM medium

Table 2. Nitric oxide concentration (mean ±SD) at different irradiation time for two types of media

Time/ media type	10min	15min	20min	25min	P value
RPMI 1640	117.9±7.85	129.4±4.35	187.5±12.5	253.7±8.3	0.001
MEM	88.5±3.5	142±22	170±0.5	220±6	0.005
P value	0.076	0.629	0.307	0.081	

In agreement with present finding, Biscop and his group (7), reported that the indirect method is affected by media type and component.

Glucose concentration after PAM treatment

Glucose uptake by cell lines was affected by plasma activated medium treatment as shown in figure 2 and table 3. First, glucose concentration was measured at different exposure time (10, 15, 20, and 25) and two incubation intervals (24 and 48 hrs). MCF7 results revealed significant column variation in both 24 and 48 with p values of (0.0009 and 0.0006) respectively, furthermore AMJ13 significant P value was <0.0001 in both

incubation intervals and finally incubating ASCs beyond 24 hrs yielded significant column alteration (p=0.002) and row P value was (0.033), whereas after 48 hrs culturing the p=0.0008 in the 48 hrs incubation.

Table 3. Glucose concentration (mg/ml) in different exposure times analyzed statistically by graph pad prism two-way ANOVA test

Cell line	10 min	15 min	20 min	25 min	Control	Row P value	Column P value
MCF7 (24hrs)	7.95	59.4	92.5	103.7	43.5	0.286	0.0006
MCF7 (48hrs)	98.5	109.5	107.8	152	11	0.685	0.0009
AMJ13(24hrs)	116.5	121.75	153.25	195.8	16	0.385	<0.0001
AMJ13(48hrs)	132.5	131.5	171.5	197	12.5	0.831	<0.0001
ASCs (24hrs)	16	22.5	26.5	36	6	0.033	0.002
ASCs (48hrs)	19	31.5	37.5	41.5	10	0.839	0.0008

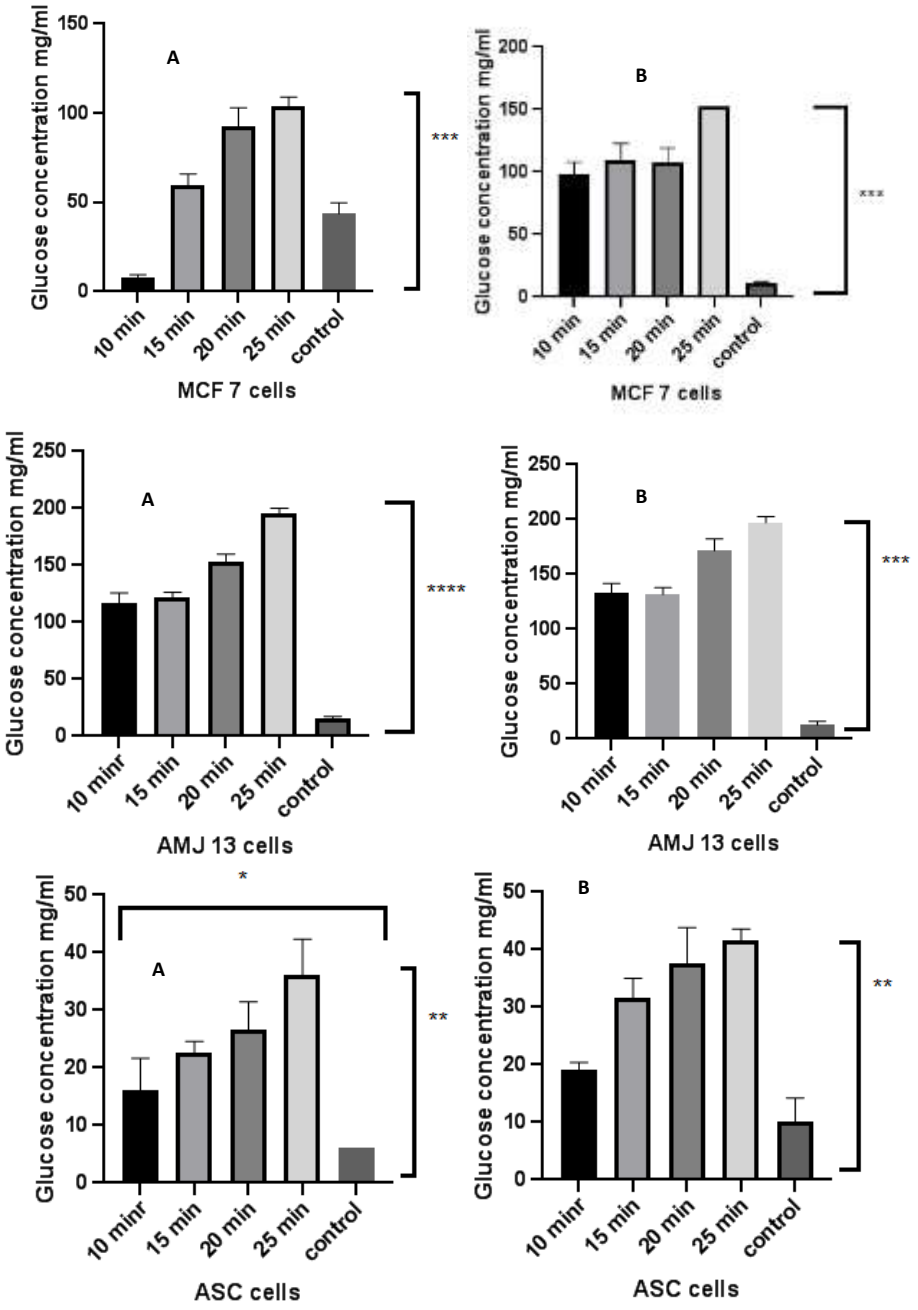

Figure 2. glucose concentration at 10, 15, 20, and25 minutes of two incubation intervals in which (A) represents 24 hours incubation while (B) represent 48 hours of incubation

Based on the results, the exposure time 20 minutes considered the best for treatment conditions. Since the glucose concentration between 20 and 25 min of incubation with plasma activated medium were non-significant and for economical prescriptive by reducing the exposure time and gas waste. Thus, the results were reinforced by treating the three cell lines for 20 min. The data of MCF7 showed significant differences between treated and non-treated cells in the first 24 hours 105± 9 mg/ml and 35.5±9 mg/ml; p=0.016. Over increasing incubation period, a stronger significant effect was demonstrated, since glucose concentration in the medium was increased in comparison to untreated cells (146.5±5.85 and 16±5.85 mg/ml; P= 0.002).In case of AMJ13 similar significant results was shown in the first day after treatment and glucose concentrations were 55± 25 mg/ml and 128±5 mg/ml in non-treated and treaded cells respectively. After 48 hours' significant alteration was generated with P value of 0.006 and the results were 17± 20.72 mg/ml and 151± 20.72 mg/ml in non-treated and treaded cells respectively, (table 4 and figure 3).

Table 4. Glucose concentration (mg/ml) in cell culture media of three cell linesgrown in treated and untreated media expressed as mean±SD

Cell line	Non treated cells (24hrs)	PAM treated cells (24hrs)	Non treated cells (48hrs)	PAM treated cells (48hrs)
MCF7	35.5±9	105±9	16±5.85	146±5.85
P value	(*)0.016		(**)0.002	
AMJ13	55±25	128.5±25	17±20.7	151.5±20.7
P value	0.040		0.006	
ASCs	0±2	13±2	0±4.5	33.5±4.5
P value	(*)0.02		(*)0.017	

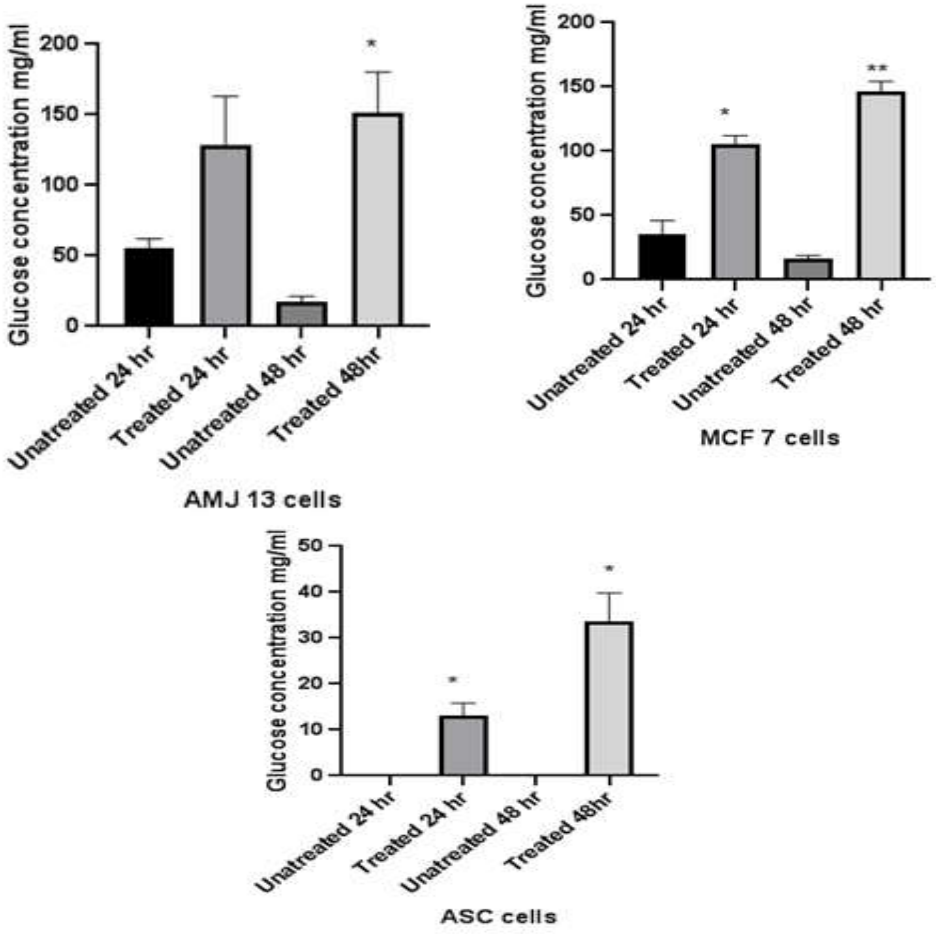

Figure 3. Concentration of glucose in culture media after 24 hrs and 48hrs incubation times

In spite there is difference in the amount of glucose amount between RPMI and MEM media (2 and 1 g/ml respectively), the results show no significant difference in glucose concentration between both cell lines either after 24 or 48 hrs (figure 4).

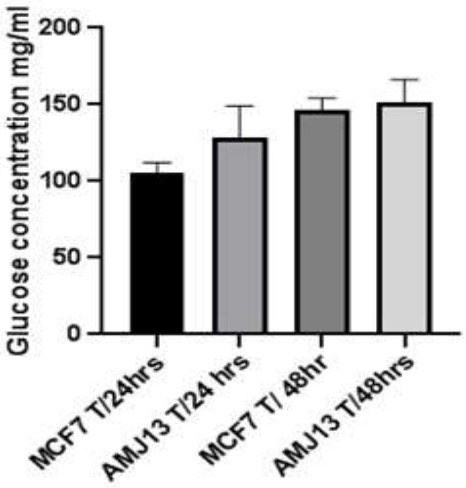

Figure 4. This figure shown the comparison between glucose level in MCF7 grown in MEM medium and AMJ13 cell line grown in RPMI-1460 medium treated for 24 and 48 hrs

Cancer cells express plenty of glucose channels on their surface enabling them to activate aerobic glycolysis and providing the cells with required energy. Adhikari and his group (1) found that the uptake of glucose in melanoma cell lines using cold atmospheric plasma decreased up to 80%. Together, these data suggest that low thermal plasma can be used to target these receptors as its capable of oxidizing the lipids in the outer membrane which result in lowering the barrier for glucose transportation across the membrane leading to a reduction in the uptake of glucose and increased its level in the medium (23). The result of glucose concentration in adipose derived stem cells glucose results indicated a significant variation in both incubations intervals and the results were (0±2 vs. 13 ±2; P=0.02) and (0 ± 4.5 vs. 33.5 ± 4.5; p=0.017) for 24 and 48 hours respectively. Based on the finding, normal non-cancerous cells (ASCs) consumed the entire glucose found in the medium under normal conditions. After plasma treatment glucose concentration was slightly elevated after a whole day of incubation. A moderate elevation was demonstrated after two days of treatment. Panina, and his colleagues (22) found that stem cells and multi-linage differentiation cells rely on glycolysis to maintain cells from uncontrolled recruitment as stem cells with adjacent cells serve as lactate source. Moreover, they documented that glycolysis determines the fate and functional activity of adipose cells.

Lactate dehydrogenase enzyme activity

The present study measured the level of LDH in both media supernatant and supernatant of cell lysate to detect if there is a potential membrane damage-causing LDH release into the media. The results of LDH level in MCF7 showed no activity in the untreated cells (supernatant from cell lysate) in both incubations hours. On the other hand, the treated cell showed a highly significant elevation in LDH after 24 hours of incubation (17.75± 0.25; p=0.003), and a significant reduction followed the 48 hrs treatment (12.75± 0.25; p=0.022). Regarding the LDH activity level in culture media, result revealed non-significant results after 24 hrs of incubation (0.03±1 and 4±1). While a significant change was recorded after48 hrs of cell incubation (3.25± 0.34 versus 8.25±0.25; p=0.008) (table 5), figure (5).

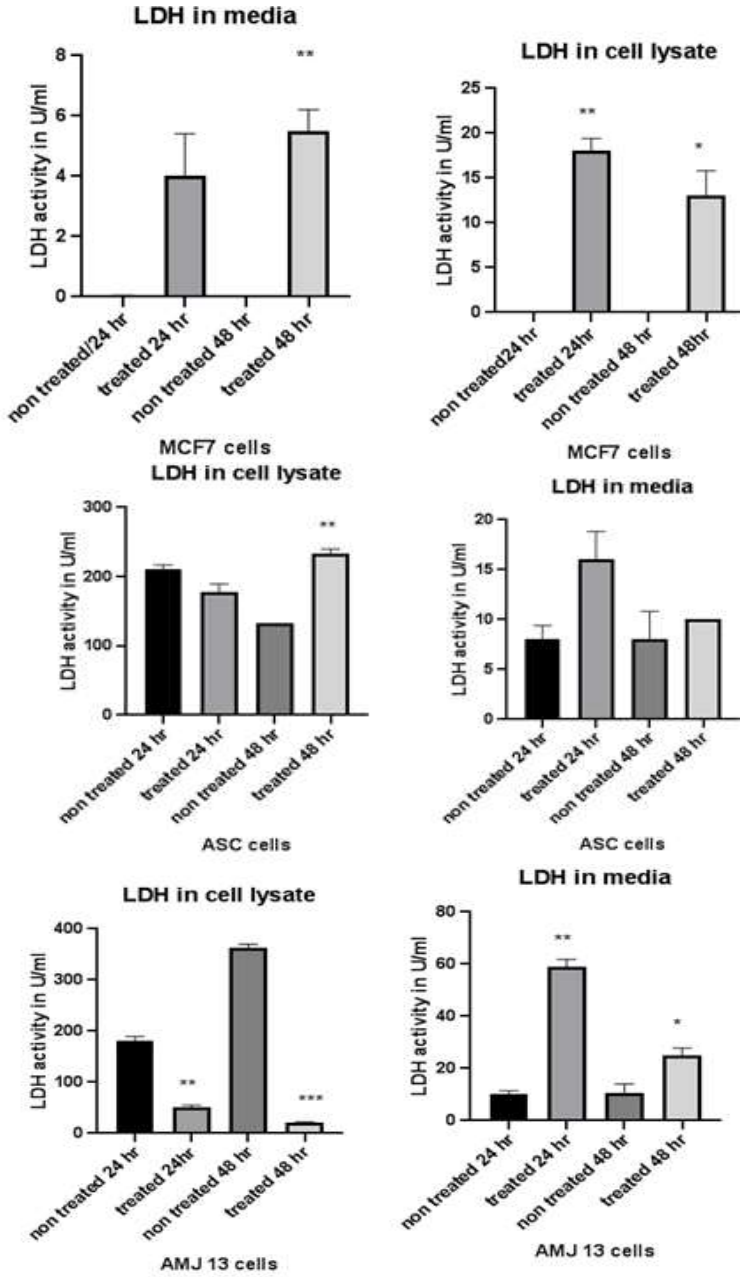

Figure 5. Lactate dehydrogenase enzyme activity in both culture media and cell lysate for MCF7, AMJ13, and ASCs and the results expressed as mean±SD, where *p≤0.05, **P≤0.001, and ***P≤0.0001

Table 5. lactate dehydrogenase activity (U/ml) in cell lysis and culture media for all studied cell lines expressed as (mean±SD)

Cell lines	24hrs untreated	24hrs treated	48hrs untreated	48hrs treated
MCF7/ cell lysate	0.001±0.25	17.75± 0.25	0.002± 0.25	12.75± 0.25
P value	(**)0.003		(*)0.022	
MCF7/ culture media	0.03±1	4±1	0.02±0.5	5.5±0.5
P value	0.058(ns)		(**)0.008	
AMJ13/ cell lysate	180± 7.4	51±7.4	363±5.22	20.5±5.22
P value	(**)0.003		(***)0.002	
AMJ13/ culture media	10±2.2	59±2.2	10.5±3.2	25±3.2
P value	(**)0.002		(*)0.045	
ASCs/ cell lysate	210±10.6	177±10.6	233.5±4.5	133±4.5
P value	0.079(ns)		0.002**	
ASCs/ culture media	8±2.2	16±2.2	8±2	10±2
P value	0.070		0.422	

It was reported that MCF7 doesn't depend on glycolysis as a source of ATP even if the glucose is the only available substrate and may use glycolysis up to 20 % when it's necessary (19). Thus it can explain the elevation of LDH after the first 24 might be due to damage in the mitochondrial membrane and blockage of oxidative phosphorylation which makes MCF7 cells shift their metabolic pathway toward the glycolysis and after 48 hours the plasma jet treatment reduces the activity of LDH. Further it was indicated that there is a relation between LDA-A and viability of MCF7 as both were reduced significantly during treatment as Al salam et al (3) mentioned. In the case of AMJ13 cells, the result demonstrated higher enzyme activity than MCF7, the present study data presented a significant reduction in enzyme activity over incubation duration corresponding to the untreated one. The 24hrs results were (180±7.4, 51±7.4; p= 0.002). While 48hrs incubation results were (363±5.22 versus 20.5±5.22; p=0.045). Furthermore, the analyzed media's supernatant indicated a significant increase in activity of the enzyme in the first 24 hrs of incubation in the control 10± 2.2 and the treated 59± 2.2 as well as the second day 10.5 ± 3.2, 25± 3.2. A conflict results were demonstrated in adipose-derived stem cells. Lactate dehydrogenase represented high activity in the cell lysate. After whole day incubation, a non-significant alteration was detected (177±10.6, 210± 10.6). While, 48 hours of incubation accompanied by significant differences (133± 4.5, 233±4.5; p=0.002). Non-significant changes were shown in both incubation times in the tested media and the results were (8±2.2, 16±2.2) after 24hrs and (8±2, 10±2) after 48 hrs respectively The findings suggested that LDH activity was decreased within the cells as the incubation time increased. The possible mechanism of cold plasma on LDH was summarized by Tolouie et al (24), they proposed that prolonged plasma treatment have the ability to alter the 3D structure of protein molecule (LDH) by decreasing α helix turns and increasing the β plated sheet, thus the secondary structure of the protein will be change and eventually, loss of function will result. While the enzyme that was released to extracellular media in the first 24 hours increases which indicates damage in the cell membrane as lactate dehydrogenase enzyme is a cytosolic enzyme that is released to the extracellular matrix only after cell membrane damage, like apoptosis or necrosis(10). Consistent study to these findings produced by (29), they treated cancer cells with cold atmospheric plasma (direct and indirect methods) and revealed that the activity of the enzyme decreases steadily in a dose-dependent manner irrespective of treatment route.

Conclusion

Plasma activated medium proved their capability to effects breast cancer cell line metabolic pathway by decreasing both glucose uptake and lactate dehydrogenase production in a dose dependent pattern. In addition, PAM overcomes the metabolic flexibility of cancer cells and eventually effects the energy production. Nitric oxide was sufficiently produced which is essential for generating the required physiological influences and its production is proportional to the exposure periods. In addition, it was found that PAM may negatively influence the metabolic program of adipose-derived stem cells through the stimulation of obesity, since it increased glucose concentration in the circulation which considered as substrate for fatty acid synthesis.

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