

CYTOTOXIC EFFECT OF PLASMA ACTIVATED MEDIUM ON THE TREATED BREAST CANCER

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ABSTRACT

This study was aimed to evaluate the effect of plasma activated medium (PAM) on breast cancer cell lines (MCF7 and AMJ13) and compared it to non malignant one (ASCs). The cytotoxic effect of 10,15,20, and 25 min prepared PAM was measured and the results indicated that all cells were influenced in the exposure times (20 and 25)min, especially after 48 hours of incubation with lesser influence on normal cells. Based on the results 20 min was determined as the effective dose on 50% of cancer cells with minimal influence on normal one. Further, tumor necrosis factor-alpha (TNF- α) was assessed and the results indicated reduction in the level of this cytokine after PAM treatment, MCF7 showed significant 2reduction in both incubation intervals while AMJ13 and ASCs significantly varied only after 48hrs.Finally, caspase-9was analyzed to identify the apoptosis route and the obtained data revealed that both MCF7 and AMJ13raisedsignificantlyafter 24 and 48hrs.In contrast, ASC cells revealed non-significant changes in both intervals.

Key words: plasma jet, caspases-9, TNF- α , and apoptosis

فاضل وآخرون

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التأثير السمي لمعاملة الخلايا الخلية لسرطان الثدي ببلازما الوسط المنشط

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المستخلص

قيمت الدراسة الحالية تأثيرالوسط المنشط بالبلازما على سلالات خلايا سرطان الثدي MCF7 و AMJ13 وقارنتها بأخرى غيرالسرطانية (ASCs). تم قياس التأثيرالسام للخلايا لبلازما الوسط المحضلمدة 10 و 15 و 20 و 25 دقيقةوأظهرت النتائج أن جميع الخلايا تأثرت بالوقت 20 و 25 دقيقةخصوصا بعد يومين من الحضان لكن كان التأثير اضعف بكثير على الخلايا الطبيعية. بناء على النتائج تم تحديد 20 دقيقة كوقت التعريض الفعال لقتل نصف الخلايا السرطانية مع اقل تأثير على الخلايا الطبيعية.اضافه الى ذلك تم قياس تأثير الوسط المنشط على تركيز TNF- α وقد اظهرت النتائج انخفاضاً مغنويا لجميع الخلايا في كلتا فترتي الحضان. واخيرا تم قياس ال كاسبيز -9 ووجد ان كلا من MCF7 وAMJ13 ارتفع بشكل ملحوظ بعد 24 و 48 ساعة،وعلى النقيض من ذلك،كشفت خلاياASC عن تغيرات غيرمهمة في كلا الفترتين.

الكلمات المفتاحية: البلازما النفاثة، عاملنخرالورمألفا، كاسبيز -9، موت الخلايا المبرمج

INTRODUCTION

Plasma activated medium (PAM) is generated by exposing the liquid medium to plasma gas. This method considered as indirect cold plasma treatment in which plasma species interact with ions and electrons in liquid and capable to provide extra reactive species through this interaction, furthermore the main activity of (PAM) depends on the long lived species that disseminate and solvate into the aqueous phase, which eliminates the effects of other molecules including: photons, electric field, short lived species, and heat (8, 11, 15, 17). Many advantages were reported over the direct method including; the liquid can be stored at room temperature or frozen (11), its effective in the treatment of deep tumors and cancers type where the physical factors are not desired like heat or UV radiation (14, 19), easy to process, store, and can be treated over a large area (13). Cancer is a worldwide disease and its one of the leading causes of death. It varies in histopathology, genetic and epigenetic characteristics (19). According to the Iraqi Cancer Registry, breast cancer is classified as the first among the cancers affecting the Iraqi population. PAM was shown to exhibit anticancer effect against multiple studied cancers such as; glioblastoma, ovarian, gastric, pancreatic, and lung cancer (12,26). One of the main mechanisms of plasma in cancer treatment is induce apoptosis, which is the main defensive strategy in higher animals that prevent cellular tumorigenic possibility by the removal of excess and harmful cells during development and pathophysiological conditions. It has a crucial role in the development and maintenance of healthy cells and proper immune function. Programmed cell death can be stimulated by a wide range of external and internal signals, such as reactive oxygen species (ROS), reactive nitrogen species (RNS) in addition to TNF- α which is a pleiotropic cytokine that plays a central role in apoptosis (4, 23,22). The main players in plasma activated medium are ROS and RNS which induce oxidative damage especially reactive oxygen species, a highly reactive molecule toward important body macromolecules including : proteins, DNA, RNA, and lipids (22,24). There are two categories of apoptosis depending on the

stimulation factor. The intrinsic apoptosis (mitochondrial apoptosis) rely on the activation of caspase-9. And extrinsic cell death is initiated via transmembrane killing receptor including TNF receptor superfamily member such as (TNFR1), after the binding of ligand to these death receptors a series of intracellular signals will activated and eventually leading to caspase-9 and 10 activation (2, 3,9, 26). The present study aimed to investigate the cytotoxic effect of plasma activated medium along with the apoptosis induction mechanism that caused the cytotoxic influence and determining the potential of PAM as new breast cancer therapy.

MATERIALS AND METHODS

Preparation of plasma activated medium

Plasma jet device was design in the laboratory of Physics Department/ University of Baghdad and supplied with argon gas and an AC power supply of 18-20 kv. The device composed of tungsten electrode covered with quartz tube of 16 cm and 0.5 in diameter. PAM was prepared by exposing two types of media (RPMI-1640 and MEM), an amount of 3ml of each media was placed in tissue culture plate with 3cm of 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 then 2 ml were added to the six well plates containing cell lines and 200 μ l was added to each well in 96 well plate. Plasma jet device was designed and supplemented from University of Baghdad/College of sciences/Department of physics.

Cell lines culturing

In the present study three cell lines were used and all were provided by the Experimental therapy department / Iraqi Center for Cancer and Medical Genetics Research (ICCMGR) Baghdad, Iraq. The first breast cancer cell line is Michigan Cancer Foundation 7 (MCF7) was cultured in MEM medium (Biochrom, German) with cell density of 1×10^4 the medium was previously supplemented with fetal bovine serum 10% (FBS; Biochrom, Germany) and 1% penicillin-streptomycin (TROGE, Germany). The second breast cancer cell line was Ahmed, Murtudha, Jabriyah, 2013 (AMJ13) (breast cancer cell

line) and the third is non-malignant cell line Adipose Derived Stem cells(ASCs) were both cultivated in RPMI-1640 (USA) at a density of 1×10^4 . 10% FBS and 1% antibiotic (penicillin-streptomycin) were added. All three cells were incubated at 37°C in 5% CO₂ (1).

Cytotoxicity assay

Crystal violet assay was used to measure the cytotoxic effect of PAM, 200 µl of cell suspension (1×10^4) were cultured within 96 well plates until reaching a confluence of 80% then they were treated with 200µl of PAM exposed medium from different exposure time(10, 15, 20, and 25 minutes) and incubated for 2 different periods 24 and 48 hour. Media were discarded and 200 µl crystal violet dye was added to each well and incubated for 20 minutes in 37°C, after that the plate were gently washed with tap water and methanol was added. The absorbance (OD) was measured at (492nm) by the ELISA plate reader(Flu star (USA)and the tumor cytotoxic ratio calculated based on the below equation:

$$\text{Cytotoxic ratio} = \frac{A-B}{A} \times 100$$

Where A = Absorbance of untreated

B= Absorbance of treated cells

Analysis of tumor necrosis factor alpha and caspases-9: Both parameters were analyzed in the cell lysate of MCF7, AMJ13, and ASC cells by conventional ELISA assay. Elabscince human TNF-α ELISA kit (USA) and Elabscince human caspases-9 ELISA kit (USA) were used, in brief the cells were cultured in 6 well plate and after treatment and incubation the cell lysate was prepared. First,

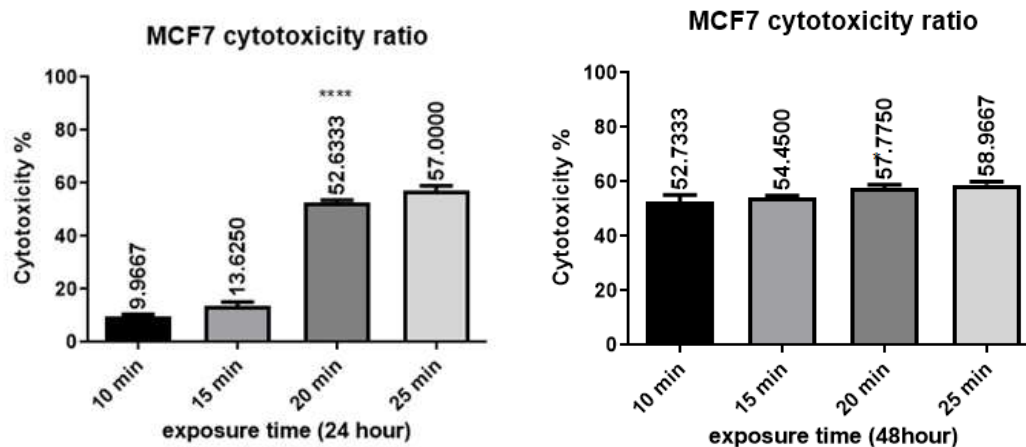
cells were collected after 1 ml of trypsin-versin(USbiological/USA)was added thenneutralized with 3ml of media, the suspension was centrifugedat10000 rpm for 10 minand the supernatant was collected. Freezing- thawing with PBS was repeated three times and centrifuged with cooling centrifuge for 10 min, the sediment was collected. The steps were performed under sterilized conditions.All Reagents were prepared according to manufacturer instruction and read at 450nm. The concentration of TNF-α and Cas-9 for the studied groups was determined according to the straight line equationresulted from blotting the optical densities of standard dilutions.

Statistical analysis

All data in this study were statistically analyzed by graph pad prism software 8 (GraphPad Software, Inc., La Jolla, CA). Column was analyzed by student t test (unpaired), the whole data expressed as mean ±standard error (SE) of at least duplicate and the differences considered significant if the value of P were below or equal to 0.05.(6).

RESULTS AND DISCUSSION

Cytotoxic ratio of plasma activated medium: The results of cytotoxic analysis showed that the plasma activated medium has a parallel toxic effect with exposure time and incubation periods in all treated cell lines (AMJ13, MCF7, and ASC)with slighter influence on the non-malignant cell line(Figure 1) and (Table 1).



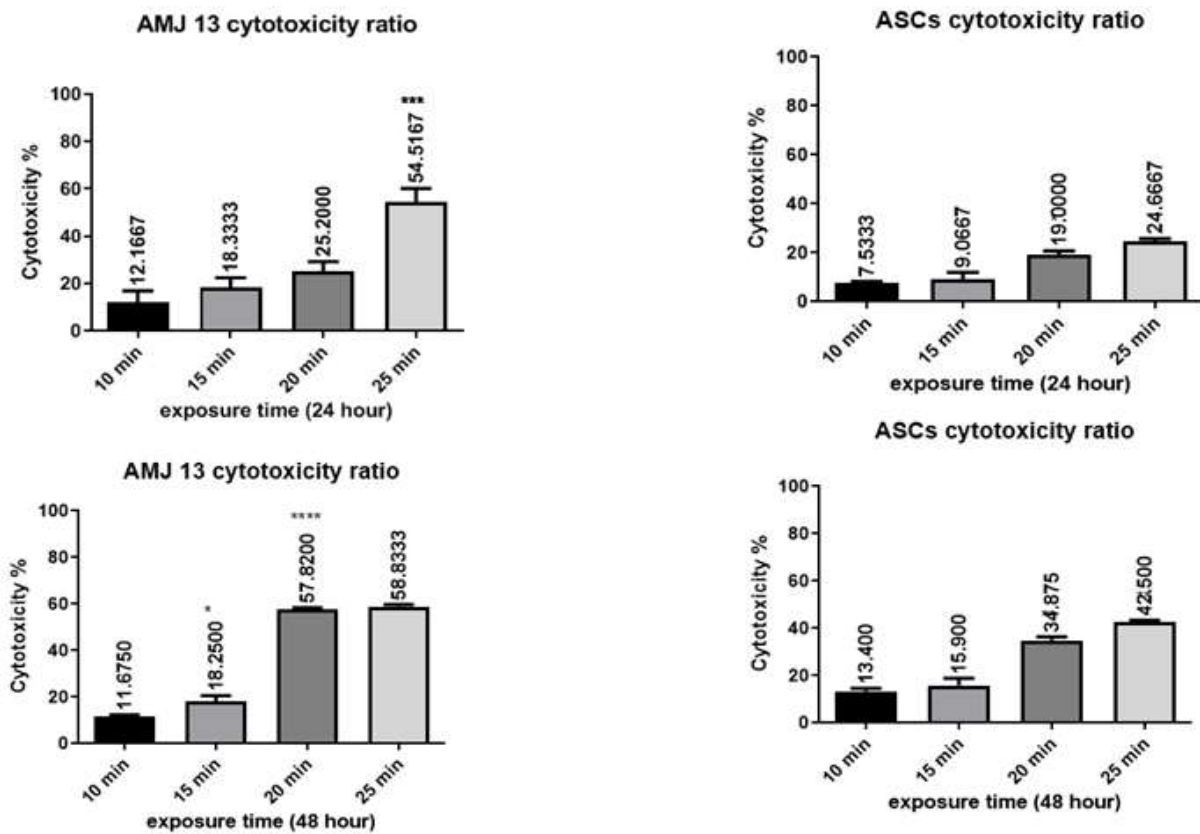


Figure 1. Cytotoxicity ratio in MCF7, AMJ13, and ASCs cell lines exposed to different time and incubated for 24 and 48 hours respectively

The effect was seen obviously on MCF7 cells, though in the first 24 hours of incubation, 10 and 15min revealed a minimal effect on cell viability with non- significant changes ($9.96 \pm 0.57\%$ and $13.63 \pm 1.6\%$) respectively. In contrast, the 20 minutes showed significant cytotoxic effect on cell viability ($52.63 \pm 0.977\%$; $p < 0.0001$) and 25 min didn't differ significantly than 20 min ($57.2 \pm 2.08\%$). The sensitivity of cells markedly elevated after 48 hrs. of incubation and the cytotoxicity ratio for 10 and 15 minutes were non-significant ($52.7 \pm 2.4\%$ and $54.45 \pm 0.5\%$). After 20 min, a significant elevation was found ($57.78 \pm 1.07\%$; $p = 0.03$) and the 25 min treatment showed non-significant differences ($58.97 \pm 1.03\%$). These results proved that the effect of plasma on cells is dose-dependent manner. Noticeable morphological changes were accompanied with PAM treatment, as the cells appeared rounded, shrank with nuclear condensation, and detached from the plate is compared to untreated cells. The number of detached cells increased with an increase in incubation period as shown in (Figure 2).

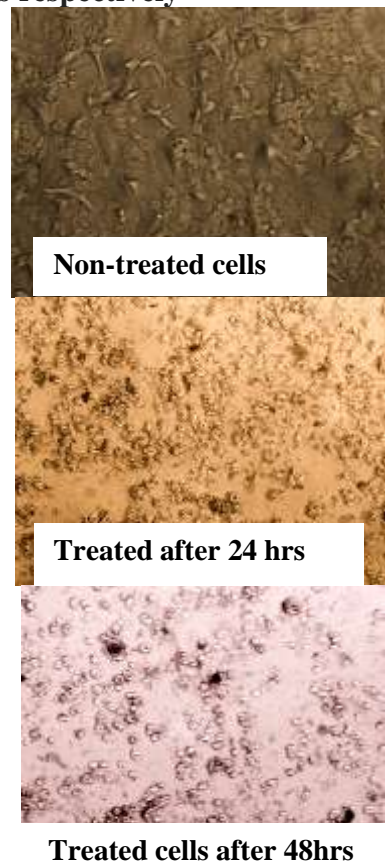


Figure 2. Morphological alteration in PAM treated MCF7 cells after 24 and 48 hrs incubation

Regarding AMJ13 cell line results, it was demonstrated that PAM had a lower influence than MCF7 cells after 24 hrs. of incubation ($12.17 \pm 2.75\%$, $18.22 \pm 2.41\%$, and $25.2 \pm 2.35\%$) the ratio of 10, 15, and 20 minutes which was statistically non-significant. After 48 hrs. a decline in cells viability was shown especially in 20 min ($57.82 \pm 0.62\%$; $p < 0.0001$) and the non-significant 25 minutes of exposure showed ($58.83 \pm 0.92\%$). While 10 and 15 minutes showed $11.68 \pm 0.73\%$ and $18.25 \pm 2.42\%$; $p = 0.040$. A contrasted result was documented considering morphological alteration since no changes were detected in AMJ13 cells (Figure 3).

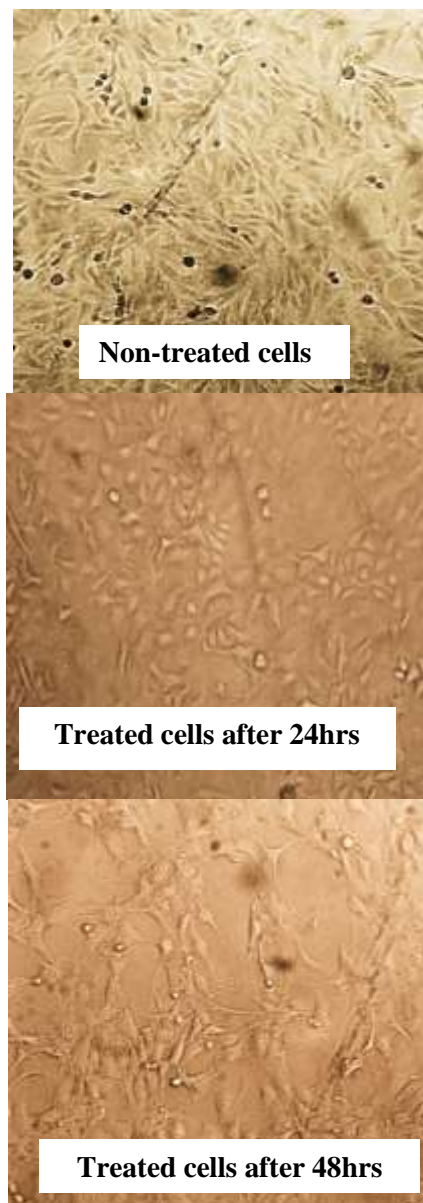


Figure 3. AMJ13 morphology after 24 and 48 hrs incubation with PAM

On the other hand, PAM had a minimal effect on adipose-derived stem cells (ASCs) in comparison to cancer cells, especially on the first day of incubation as the inhibition ratios were ($5.87 \pm 1.74\%$, $10.83 \pm 2.72\%$, $21.44 \pm 1.79\%$, and $25.7 \pm 1.28\%$) the result was significant only for 20 min with p value of 0.045. A gradual inhibition in the viability of cells rise with the prolonged incubation beyond 48 hours and the growth inhibition ratios were ($13.4 \pm 1.27\%$, $15.9 \pm 2.79\%$, $31.17 \pm 1.3\%$, and $39.23 \pm 0.66\%$) for 10, 15, 20, and 25 min. respectively. The results were significant for both 20 min treatment ($p = 0.001$) and following 25 min exposure ($p = 0.006$). Similarly, there was no observed morphological change in ASCs in treated cells at both incubation periods. As shown in (Figure 4).

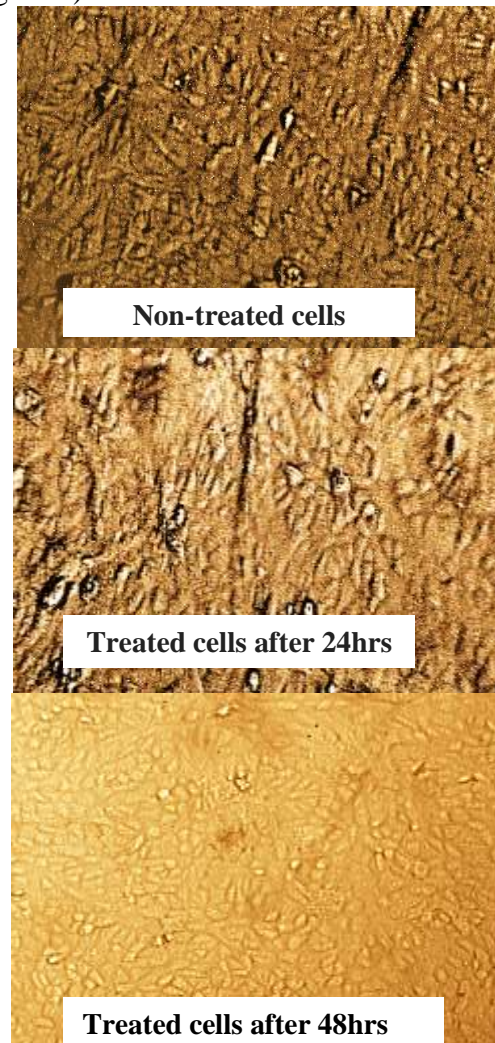


Figure 4. PAM effect on non-malignant cells morphology after 24 and 48 hrs incubation

Table 1. This table illustrates the cytotoxic ratio (mean± SE) on different cell lines incubated with PAM for 24 and 48 hours respectively

Cell lines i	10 min	15 min	20 min	25 min
MCF7/ 24 hrs.	9.97±0.58%	13.63±1.6%	0.11(ns)	52.63±0.97%
MCF7/ 48 hrs.	52.7± 2.4%	54.45± 0.5%	0.45(ns)	57.78± 1.07 %
P-value	<0.0001***	<0.0001***	0.019*	0.030*
AMJ13/24 hrs.	12.17± 2.75%	18.22±2.41%	0.16(ns)	25.2± 2.35%
AMJ13/48 hrs.	11.68± 0.73%	18.25± 2.42%	0.041*	57.82± 0.62 %
P-value	0.84(ns)	0.98(ns)	<0.0001***	0.11(ns)
ASC/ 24 hrs.	5.87± 1.74%	10.83± 2.72%	0.46(ns)	21.44± 1.79 %
ASC/ 48 hrs.	13.4± 1.27%	15.9± 2.79%	0.48(ns)	31.17± 1.3%
P-value	0.016*	0,17(ns)	0.001**	0.001**
				<0.0001***

Regarding the possible mechanism of PAM cytotoxicity, recent studies proposed that the reactive agents are responsible for the selective killing of PAM [as cancer cells typically have higher resting levels of ROS than normal cells], and the additional RONS provided by PAM will raise these species to toxic levels in cancer cells through stimulating the production of intracellular RONS and enhance apoptotic pathway (10, 29). From the present results, important observations have to be highlighted with respect to PAM treatment; the first is the anti-tumor selectivity of CAP which was reported and demonstrated for over 20 cancers type and their effect on breast cancer revealed high selectivity and sensitivity in comparison to normal cells without or with minimal side effects, even the animal models (*in-vivo*) studies showed that it has a selective apoptotic influence toward cancer cell leaving the normal cells non touched which make PAM a promising oncotherapy for many cancers including breast cancer (11, 31). The present result was reinforced by Ly and his colleagues (18) they applied PAM on all types of breast cancer cell lines and determined that, it has effect on the viability of all cell lines. As well as, Terefinko and his coworker (28) have similar results on the first and second day of PAM incubation and they suggested that the reduction of cell viability is a result from high RONS level which impact the cells and chemical component of media used. Thus, according to the cytotoxic effect of PAM, 20 min. was the best selected time and the further experiments were depended on.

TNF- α response to PAM

Based on the importance of this inflammatory mediator in the survival and/or inhibition of breast cancer (20), the effect of PAM on TNF- α was examined in breast cancer cell lines and non-malignant one. The results demonstrated a significant decrease in the concentration of this mediator in both incubation time (24 and 48 hrs.) in comparison to non-treated homologue over incubation periods in MCF7 (17.28±3.5 versus 39.4±2.6; p=0.037 after 24 hrs.) and (25.14±2.8 versus 48±2.5 ; p=0.026 after 48hrs. treatment). Whereas, local breast cancer cell line results suggested less influence by PAM treatment especially after whole day incubation (5.85±0.71 versus 6.76±1.04) which showed non-significant alteration. Though after 48 hrs. a significant reduction in the level of TNF- α appeared in treated cells versus the untreated cells (7.1±1.65 versus 15.75±0.25; p=0.035). Similar action of PAM was demonstrated on adipose-derived stem cells as the concentration of TNF- α decreased in both 24 and 48 hrs. Still, it was found that the concentration of the latter parameter was very high in the untreated ASCs in comparison to MCF7 and AMJ13 cells. Following the incubation for 24 hrs. concentration dropped non-significantly from 306.1± 10.3 to 263.2± 5.66. While, after two days of treatment TNF- α declined significantly in treated cell corresponds to the untreated (391±6.4 vs 278±0.025; p=0.023) (Table 2 and Figure 5).

Table 2. TNF- α concentration (pg/ml) represented as mean \pm SD for different cell lines at two incubation periods

Cell lines	Untreated cells/24hrs.	Treated cells/24hrs.	Untreated cells/48hrs.	Treated cells/48hrs.
MCF7	39.4 \pm 2.6	17.28 \pm 3.57	48 \pm 2.5	25.14 \pm 2.8
P- value		0.037(*)		0.026(*)
AMJ 13	6.76 \pm 1.04	5.85 \pm 0.71	15.75 \pm 0.25	7.15 \pm 1.65
P- value		0.547(ns)		0.035(*)
ASCs	306.1 \pm 10.3	263.2 \pm 5.66	391.4 \pm 6.4	278 \pm 0.02
P- value		0.067(ns)		0.023(*)

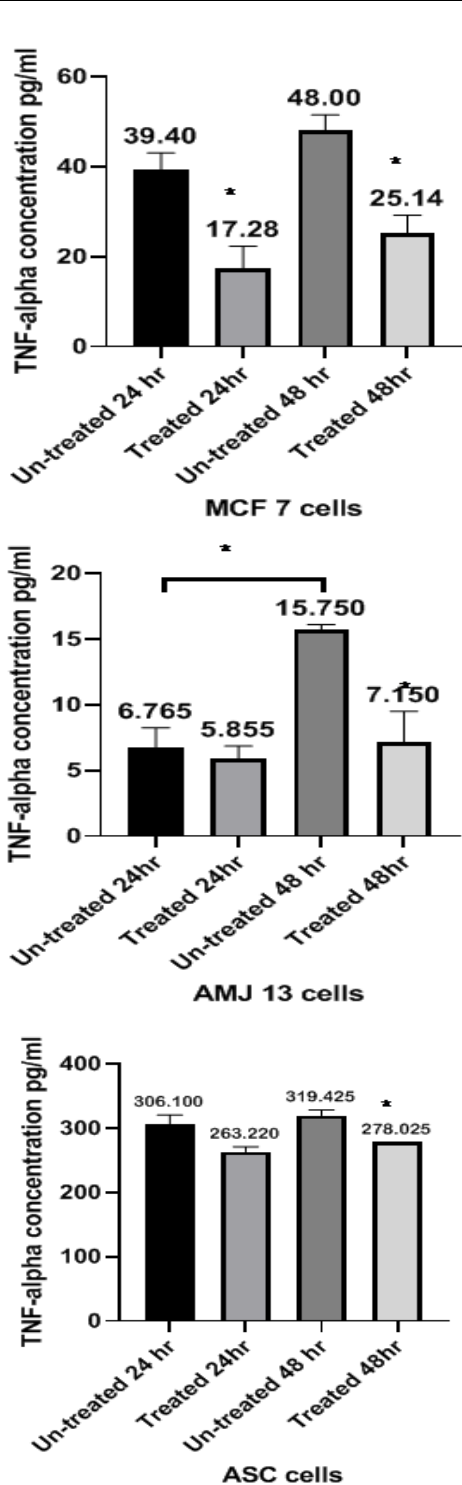


Figure 5. Tumor necrosis factor alpha concentration pg/ml

Based on the collected data and previous studies, it can be suggested that plasma activated medium had inhibitory effect on TNF- α in all treated cells and incubation periods subsequently, it effected the proliferation and survival of cells as many studies reported the importance of this mediator in breast cancer proliferation and invasiveness. As it was mentioned by Berberogluet al.,(7)that TNF- α reduced in serum of breast cancer patients after chemotherapy at multiple stages of the disease. Regarding the variation between AMJ13 and MCF7 level of TNF- α might be likely due to the origin of the cell line whether it was from metastatic tumor or not as this inflammatory cytokine associated with the invasiveness of the tumor and migration capacity as it was mentioned by An et al., (5) that (TNF- α) elevated in the serum of breast cancer patient at the second and third stages along with patients of lymph node spreading. Further study emphasized the present study which elucidated that low thermal plasma had an inhibitor influence on TNF- α associated nuclear factor kappa (NF- κ B) activation, suggesting anticancer activity. As well it was the intracellular reactive species generated in response to cold atmospheric plasma treatment may likely stimulate the expression of apoptotic genes mediated by this cytokine (35). Alternatively, it was reported that at low doses of CAP (10 sec) increased the secretion of TNF- α with other cytokines and it was acclaimed that the cytotoxicity of this inflammatory mediator is dose dependent as a result of cytokine signal alteration between cells (21). Considering local adipose-derived stem cell results, high level of TNF- α considered predictable as this cytokine synthesized by adipose tissue and it was reported that TNF- α level increase 2-fold in response to an increase in the size of

adipocyte, such increase in size is accompanied by obesity (16).

Apoptosis and caspase- 9 activation in response to PAM: Enzyme linked Immuno Sorbent Assay was used to assess the presence of caspase 9 in PAM treated cells (AMJ13, MCF7, and ASCs). A significant elevation in the CAS-9 was seen after 24 and 48hrs. of MCF7 treatment in compared to non-treated cells (56.25 ± 1.75 versus 6.65 ± 1.55 ; $p=0.002$ and 48.5 ± 0.5 versus 7.5 ± 0.4 ; $p=0.0002$) respectively. Similar data were found in AMJ13 cells, significant elevation reported in both incubation intervals. After 24 hrs. the results were 5.85 ± 1.05 ($p=0.002$) for untreated and 48.25 ± 1.95 ($p=0.031$) for treated group, after 48 hrs. the results were 6.8 ± 1.3 and 34.5 ± 2.5 . Alternatively, ASC cells revealed non-significant changes in all treated and non-treated groups (12 ± 2 and 14.5 ± 0.5), (15 ± 3 and 11.5 ± 2.5) respectively for 24 and 48 hours, (Figure 6 and Table 3). Yang et al., (34) found that plasma activated solution led to significant elevation in both Cas9 and Cas3, suggesting intrinsic apoptotic pathway accompanied by non- significant changes in normal cells. Otherwise a drop in the cleaved caspase possibly due to the necrosis of cells Based on the collected result it can be suggested that PAM activated the intrinsic apoptosis pathway (mitochondrial) in treated cancerous cells in compared to non-treated cells and normal cells. A consistent study indicated that mitochondrial apoptotic pathway mediated by capases-3 and 9 was significantly activated after 30min incubation with plasma activated medium through a series of reactions begin with the inhibition of protective membrane catalase by singlet oxygen followed by elevation of hydrogen peroxide efflux (H_2O_2) though aquaporines and cytochrome C release. Finally, lead to cell death by apoptosis activation, this process will keep low H_2O_2 level in the environment which maintains normal cells unharmed (25). Another emphasized study produced by Yan et al., (33) reported that apoptosis is the main death pattern triggered by non-thermal plasma treatment and it was found that cancer treated with CAP express caspase-9 to after releasing cytochrome C to the cytoplasm. The authors added that CAP followed the known apoptotic

pathway steps from mitochondrial damage to DNA damage and cell death. In contrast, Xu et al., (32) analyzed caspases 3,8, and 9 in cell treated with CAP and they reported that all caspases started to elevate after six hours and the highest caspases was cas 8 and it was responsible for apoptotic pathway activation due to plasma treatment. Regarding adipose-derived stem cells, it can be explained based on Wang et al., (30) whom found that caspase-9 was expressed in adipose derived stem cells and it's located in the cytosol around the nucleus.

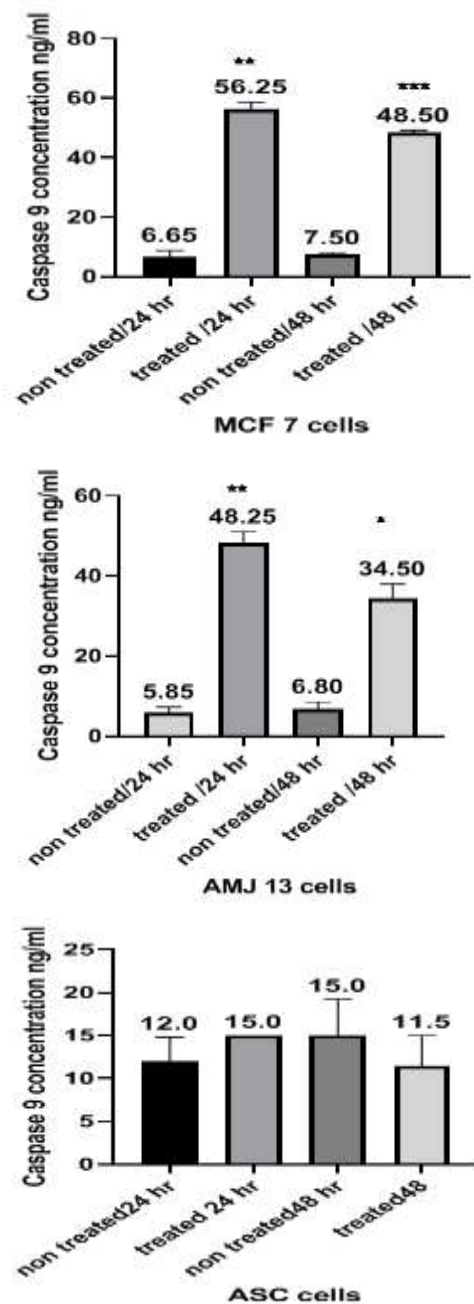


Figure 6. Caspase-9 level (ng/ml) after plasma activated incubation with chosen cells (MCF7, AMJ13, and ASCs)

Table 3. Analyzed caspase-9 level (mean ±SE) in studied cell lines

Cells	Untreated 24hrs.	Treated 24hrs.	Untreated 48hrs.	Treated 48hrs.
MCF7	6.65±1.55	56.25±1.75	7.5±0.4	48.5±0.5
P-value		0.002(**)		0.0002(**)
AMJ13	5.85±1.05	48.25±1.95	6.8±1.3	34.5±2.5
P-value		0.002(**)		0.031(*)
ASCs	12±2	14.5 ±0.5	11.5±2.5	15±3
P-value		0.272(ns)		0.464(ns)

CONCLUSIONS

It can be concluded that plasma activated solution have an obvious cytotoxicity on cancer cells viability, since the influence of PAM started after 24 hrs or maybe earlier. It's important to notify that PAM effect is a dose dependent as the activity increased after 20 min treatment and 48 hours incubation an increasing the exposure revealed an influence on the normal cells especially after 25 minutes. Elevation in cas9 accompanied with reduction in TNF- α which indicate an activation of intrinsic apoptotic pathway.

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