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Promising role of Metformin in reducing the viability of breast cancerous cells

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Abstract: To evaluate the anticancerous effects of different dilutions of metformin were evaluated for *in vitro* anti-cancerous effects, primarily breast cancer cells (MCF-7, MDA-MB-231). This prospective experimental study was conducted in Department of Pharmacology & Therapeutics BMSI in alliance with PCMD. The duration of study was from March 2016 to February 2017. For evaluating the anticancerous effects of different dilutions of Metformin (0.5µM -100µM) we used 4 different cancerous cells lines; MCF-7, HT-29, MDA-MB-231 and Hela. For assessment of anticancerous effects we used MTT assay by which assessed IC50, SI, % viability of all cells and Trypan blue exclusion assay for only MCF-7 cell line. The % viability of MCF-7 was significantly decreases (χ^2 (2) = 26.48, p=<0.001) in dose dependent manner from 99.8±0.2 to 39.71±1.3. For MDA-MB-231% viability significantly reduced (χ^2 (2) =26.48, p=<0.001) from 99.474± 0.298 to 51.55±4. However Metformin had statistically no significant dose dependent effects on % viability of MCF-10 (χ^2 (2) = 11.709, p=0.069). Metformin significantly exhibited the anticancerous effects on breast cancerous cells by selectively target the cancerous cells without any effects on normal epithelial cells of breast.

Keywords: Metformin, i*n vitro*, cancerous cell lines, % viability, viability assays, breast cancer.

INTRODUCTION

Metformin in belongs to biguanide group that was safer economical option for treatment of hyperglycemia in type 2 diabetic mellitus because of its euglycemic effects (Irons and Minze, 2014). Beside of its primarily therapeutic effects in DM, it also has beneficial effects in other conditions. Such as improve fertility in PCOS (poly cystic ovarian disorder) (Lashen, 2010), reducing the levels of LDL and cholesterol thus minimizing the chances of cardiovascular events associated with hyper chlosteremia (Xu *et al*., 2015). In addition, Metformin users of diabetic patient has lower chances of open angle glaucoma (Lin *et al*., 2015), lesser incidence of sleep apnea (Ramadan *et al*., 2006) and also have possibly promising effects on bone density (Sundararaghavan *et al*., 2017).

Aside from previously mentioned benefits as of late most vital promising intensifying both remedial and preventive part of impacts of Metformin in malignancies particularly breast (Grossmann *et al*., 2015), cervical and endometrial carcinomas (Kasznicki *et al*., 2014) Metformin can exert their anticancerous effects both as direct (insulin independent effects) and indirect effects (insulin dependent effects)(Camacho *et al*., 2015).

Insulin plays a pivotal role in oncogenesis and prognosis especially of breast cancer, by having proliferating and anti-apoptotic effects (Ryu *et al*., 2014, Paul and Mukhopadhyay, 2004). Hence metformin may be able to decreases the progression of cancer especially by reducing both insulin circulatory levels and Insulin Receptor (IR) expression (Viollet *et al*., 2012).

Most importantly metformin exerted insulin independent effects by activating AMPK pathway which in turn inhibiting the mTOR pathway (Lauretta *et al*., 2016). mTOR pathway plays an imperative share in pathogenesis of cancer by means of fulfilling the nutritional requirements of cancerous cells by increasing the expression of proangiogenic growth factors (such as VEGF, PDGF), increasing the expression of nutritional transporters over the cell membrane and also promoting cellular growth cycle by increasing the synthesis of Cyclin D1(Cargnello *et al*., 2015).

Our objective was to evaluate more debatable and emerging anticancerous effects of metformin primarily on breast cancerous cell lines. MCF-7 cell line (Invasive Ductal Carcinoma) is representative of Luminal A in accordance to molecular classification of breast carcinoma (Mota *et al*., 2017). This type of cell is hormonal receptors positive (ER+, PR+) but HER2-. These types of cells mostly respond to hormonal therapy and also often chemotherapeutic agent responsive (Dai *et al*., 2017).

On the other hand MDA-MB-231 cell lines have astonishing invasive and metastatic potential and representative of triple negative in accordance to molecular classification of breast carcinoma. Because these type of cells do not express any receptors such as ER-, PR- and HER2-(Dai *et al*., 2017).

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MATERIALS AND METHODS

This *in vitro* trial conducted in department of Pharmacology & Therapeutic of BMSI in alliance with PCMD. The ethical committee of JPMC approves this protocol. For assessment of antitumor activity of metformin we used four cancerous cells designated MCF-7, HT-29, MDA-MB-231, Hela besides that non-cancerous cell representing the normal epithelial cell of breast tissue (MCF-10).

These cancerous cell lines were Cultured DMEM which stand for Dulbecco's Modified Eagle's Medium (Modified form of basal Eagle Medium) supplemented with antibiotics (Penicillin, Streptomycin), Antifungal (Amphotericin B), Fetal Bovine serum (FBS), Sodium Pyruvate and at 37^{o}C in a humidified atmosphere (Dai *et al*., 2017).

Cell culture of all cell lines had been incubated with distinctive dilutions (as a minimum 6 dilutions) of Metformin (0.1mM -100mM) (beginning from decrease dilution) for 48-72 hours. As protocol described by means of Cumming *et al*. 2007(Cumming *et al*., 2007) for in-vitro trial total 28 samples have been analyzed for each examine cell line (4 tests for every day for each dosage hence to 4 days). For all studied cell lines viability inhibition have been assessed through MTT assay which stands for "3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay". Moreover for MCF-7 cellular line cytotoxicity was also appraised through Trypan blue dye exclusion assay.

MTT assay become a clorometric check that assessed the viability of cells with the aid of based of major that necrosis or apoptosis result in reduction of cellular viability and reduction of MTT reagent can handiest occur in metabolically energetic cells that measured spectophometrically. MTT assay variables covered absorbance values of blank (Ab), control (Ac) and test (At). From those values we calculated % viabilities and 'fa' value of studied cellular strains (Ciapetti *et al*., 1993).

Trypan blue dye exclusion assay was alternative comprehensively used cytotoxicity assay that was used for comparing the quantity of number of cells that are viable with death cells in the studied cellular suspension. It's primarily founded on principle that feasible cells that confine the doorway of colored dye (Trypan blue) inside to the cells owing to their integral plasma membrane on the contrary lifeless cells do no longer because of loss of integrity of cellular membrane (Stoddart, 2011).

The IC50 specified the drug concentration required to decrease the quantity or else fraction of cells near 50% in comparison to controls (Sliwka *et al*., 2016). Selectivity index of drug was calculated by using dividing the IC50 of the nonmalignant cells by means of that within the cancerous cells (Patel *et al*., 2009).

STATISTICAL ANALYSIS

Statistical Analysis was done through statistical software SPSS (Statistical Package of Social Sciences) version 21.0. The results were analyzed by using Kruskul-Wallis to estimate the dose dependent effects of Metformin on cancerous cell lines. A p- value <0.05 was considered as statistically significant.

We used Kruskal - Wallis test for statistical analysis because this statistical analysis method best for biological experimental cell cultured based trials (Hami *et al*., 2017). In our study for evaluation of growth inhibitory effects of metformin we performed cytotoxic assays MTT and Trypan blue dye exclusion assay. Primarily for MTT assay we performed readings four times and each day we performed readings quadruply. Although we assessed effects on same cell line but on different batches of cell cultured that's why we evaluated statistical analysis of our data by non-parametric statistical test (Tomankova K *et al*., 2015).

RESULTS

Metformin significantly reduced the % viabilities of MCF-7 ($^{\chi 2}$ (2) =26.48, p=<0.001) and MDA-MB-231 (χ^2 (2) =26.48, p=<0.001) cell lines in dose dependent manner. However metformin unable to decreases the % viability of MCF-10 (χ^2 (2) =11.709, p=0.069). As depicted in table 1, 2 and 3. Metformin was more effective in MCF-7 cell line as displayed by lowest IC50 value. As shown in table 4.

Metformin significantly reduces the viable cell counts and along with % viability in dose dependent manner of MCF-7 as appraised via Trypan blue dye exclusion assay. As shown in table 6.

DISCUSSION

Breast cancer is one of the foremost challenging problems globally mostly for the part of its diagnosis, prevention and for its therapeutic options (Wardle *et al*., 2015). As of now the mortality of breast malignancy is as yet expanding generally in view recently due to late diagnosis and poor compliance of patients. The basic reasons for poor compliance identified with anticancerous drugs were because of financial issues, genuine unfavorable impacts and anxiety of parenteral therapy (Dey, 2014). That is the reason now a days there is more concentrate on analysis of efficient oral economical treatment which was compelling against cancerous cells (Crawford , 2013).

Malignancy is a multifactorial due to its association with hereditary, ecological and metabolic disorders (Wilcox, 2005). One of the distinguishing highlights of malignancy cells is unhindered proliferation and everlasting status. Several growth factors assume an imperative part in initiation of proliferation of cancerous cells. Out of which one of the main growth factor is IGF-I (insulin-like development factors) which displays dual mitogenic and antiapoptotic impacts. The greater part of the IGF-I in the flow is created by the liver and is bound to insulin-like development factor restricting proteins (IGFBPs) (Crawford, 2013).

An elevated level of circling insulin diminishes levels of insulin like development factor restricting protein, in this manner expanding the IGF-1 (Jehle *et al*., 2003). Compensatory hyperinsulinemia because of insulin protection could prompt development straightforwardly or in a roundabout way by expanding the levels of other more powerful growth factors (IGF) or it can make cells more delicate to other development factors. So expanded insulin levels and insulin resistance may assume a part in the advancement and progression of cancer (Wilcox, 2005).

Table 1: Comparison of dose dependent effects of metformin on MCF-7 cell line viability evaluated by MTT assay

Doses (µM)	N=28	Variables				
		Ab' Mean ± SD	At Mean ± SD	Ac Mean ± SD	% Mean ± SD	Fa Mean ± SD
0	4	3.8 ±0.5	0.27 ± 0.01	0.26 ±0.01	99.8 ±0.2	0.002 ±0.002
		(3.0 -4.0)	(0.26 - 0.28)	(0.25 -0.28)	(99.6 -100)	(0 -0.004)
0.5	4	3.8 ±0.4	0.24 ± 0.008	0.26 ±0.008	89.13 ±1.5	0.11 ±0.015
		(3.5 -4.5)	(0.23 - 0.24)	(0.26 -0.28)	(87.98-91.38)	(0.09 -0.12)
1	4	4.3 ±0.8	0.21 ± 0.007	0.26 ±0.009	78.9 ±1.9	0.23 ±0.04
		(3.3 -5.3)	(0.2 - 0.21)	(0.25 -0.28)	(76.9 -81.4)	(0.19 -0.28)
1.5	4	3.2 ±0.2	0.18 ± 0.007	0.26 ±0.009	69.32 ±2.6	0.35 ±0.08
		(2.0 -4.0)	(0.18 - 0.19)	(0.25 -0.27)	(67.25-72.73)	(0.27 -0.46)
2	4	3.3 ±0.2	0.15 ± 0.008	0.26 ±0.008	58.66 ±1.8	0.48 ±0.141
		(3.0 -3.5)	(0.15 - 0.16)	(0.25 -0.27)	(57.1 -61.23)	(0.39 -0.69)
2.5	4	3.1 ±0.1	0.13 ± 0.007	0.26 ±0.008	49.34 ±2	0.51 ±0.032
		(3.0 -3.3)	(0.12 - 0.14)	(0.25 -0.27)	(46.78-51.58)	(0.47 -0.54)
5	4	3.4 ±0.5	0.10 ± 0.007	0.26 ±0.009	39.71 ±1.3	0.52 ±0.173
		(2.8 -4.0)	(0.1 - 0.11)	(0.25 -0.27)	(38.53-41.55)	(0.26 -0.61)
P-value		0.075	<0.001**	0.786	<0.001**	<0.001**

'Mean ± SD in x10-3, '(Min - Max) in x10-3, N=4 samples per day for each dose for 4 days so N=16 but Data analysis were done after entering mean value for each dose for each day so N became 4 for each dose and Total N=28 for individual drug, **Significant at1%

Table 2: Dose dependent consequences of metformin on MDA-MB-231 cell line viability evaluated via MTT assay

Doses (µM)	N=28	Variables				
		Ab' Mean ± SD	At Mean ± SD	Ac Mean ± SD	% Mean ± SD	Fa Mean ± SD
0	4	4.2 ±0.1	0.33 ±0.015	0.32 ±0.018	99.474 ± 0.298	0.005 ± 0.0003
		(4 -4.3)	(0.32 -0.35)	(0.31 -0.35)	(99.15– 99.87)	(0.0013-0.008)
0.5	4	4.1 ±0.7	0.3 ±0.017	0.33 ±0.014	91.38 ±1.3	0.09 ±0.013
		(3.3 -5)	(0.29 -0.33)	(0.32 -0.35)	(90 -93.13)	(0.07 -0.1)
1	4	4.3 ±0.3	0.28 ±0.019	0.33 ±0.015	83.43 ±2	0.17 ±0.02
		(4 -4.8)	(0.26 -0.3)	(0.32 -0.35)	(81.28 -86.05)	(0.14 -0.19)
1.5	4	4.4 ±0.2	0.25 ±0.021	0.33 ±0.015	74.21 ±2.9	0.26 ±0.029
		(3.3 -5.5)	(0.23 -0.28)	(0.32 -0.35)	(71.93 -78.43)	(0.22 -0.28)
2	4	4.3 ±0.8	0.22 ±0.022	0.33 ±0.015	66.38 ±3.6	0.34 ±0.036
		(3.3 -5)	(0.2 -0.25)	(0.31 -0.35)	(63.53 -71.6)	(0.28 -0.36)
2.5	4	4.4 ±0.1	0.2 ±0.017	0.33 ±0.017	59.2 ±2.4	0.41 ±0.028
		(4.3 -4.5)	(0.18 -0.22)	(0.31 -0.35)	(56.65 -62.1)	(0.37 -0.43)
5	4	3.8 ±0.6	0.17 ±0.015	0.33 ±0.017	51.55 ±4	0.48 ±0.04
		(3 -4.5)	(0.16 -0.19)	(0.31 -0.35)	(45.88 -54.98)	(0.45 -0.54)
P-value		0.716	<0.001**	0.959	<0.001**	<0.001**

'Mean ± SD in x10-3, '(Min - Max) in x10-3, **Significant at1%

Hence metformin can reverse the hyperinsulinemia and also reduces the expression of tyrosine kinase associated Insulin receptors. So metformin can efficiently reduce the progression and development of several cancers associated with hyperinsulinemia specially breast cancer (Giovannucci *et al*., 2010). Apart from that metformin via AMPK activation can inhibits the activity of mTOR and thus hindrance the progression of cancer (Zhao *et al*., 2017). Additionally metformin have direct cytotoxic effects on cancerous cells via activation of apoptotic pathways via both caspase and poly (ADP-ribose) polymerase (PAPP) dependent mechanisms (Zhuang and Miskimins, 2011).

In this study metformin cause admirably reductions of viability of MCF-7 as signposted by significantly diminishes the % viability (χ^2 (2)=26.48, p<0.001) as evaluated by means of MTT assay in dose dependent

Table 3: Comparison of dose dependent effects of metformin on MCF-10 cell line viability evaluated via MTT assay

Doses (µM)	N=28	Variables				
		Ab' Mean ± SD	At Mean ± SD	Ac Mean ± SD	% Mean ± SD	Fa Mean ± SD
0	4	3.8 ± 0.8	0.479 ± 0.007	0.48 ± 0.007	99.621 ± 0.228	0.0038 ± 0.0023
		(3 - 4.8)	(0.471 - 0.489)	(0.47 - 0.49)	(99.414 - 99.94)	(0.00-06 - 0.0058)
20	4	4.3 ± 0.5	0.478 ± 0.007	0.48 ± 0.006	99.489 ± 0.301	0.0051 ± 0.0030
		(3.8 - 5)	(0.471 - 0.488)	(0.48 - 0.49)	(99.043 - 99.601)	(0.0031 - 0.0096)
30	4	4.7 ± 0.2	0.478 ± 0.006	0.48 ± 0.005	99.704 ± 0.292	0.0030 ± 0.0029
		(4.5 - 5)	(0.471 - 0.486)	(0.47 - 0.49)	(99.297 – 99.937)	(0.0006- 0.007)
45	4	4.3 ± 0.5	0.478 ± 0.007	0.48 ± 0.006	99.573 ± 0.382	0.004 ± 0.0038
		(4 - 5)	(0.469 - 0.485)	(0.47 - 0.49)	(99.042 – 99.936)	(0.0006 - 0.0096)
55	4	4.7 ± 0.7	0.476 ± 0.007	0.48 ± 0.006	99.396 ± 0.394	0.006 ± 0.0039
		(4 - 5.3)	(0.467 - 0.484)	(0.47 - 0.49)	(98.995 – 99.853)	(0.0015 - 0.010)
65	4	4.2 ± 0.6	0.474 ± 0.006	0.48 ± 0.006	99.108 ± 0.048	0.0089 ± 0.00048
		(3.5 - 4.8)	(0.467 - 0.481)	(0.47 - 0.49)	(99.044 – 99.148)	(0.0085 - 0.0096)
75	4	4.3 ± 0.4	0.472± 0.006	0.48 ± 0.006	99.058 ± 0.70	0.0094 ± 0.0007
		(3.8 - 4.5)	(0.465 - 0.478)	(0.47 - 0.48)	(98.998 – 99.159)	(0.0084 - 0.010)
P-value		0.381	0.668	0.970	0.069	0.069

'Mean ± SD in x10-3, '(Min - Max) in x10-3

Table 4: Comparison of IC_{50} values of metformin among all treated cells

Cell lines N=5	IC50 Mean ± SD	P-Value
MCF-7	1.6 ± 0.1	< 0.001**
	(1.6 - 1.7)	
MDA-MB-231	2.3 ± 0.5	
	(1.9 - 2.9)	
HT-29 human colorectal adenocarcinoma	8.4 ± 1.7	
	(7.0 - 10.9)	
Hela cell line	6.0 ± 1.0	
	(4.8 - 7.3)	
MCF-10	530.9 ± 66.3	
	(476.6 - 619.4)	

Mean ± SD, (Min - Max), **Significant at 1%

Table 5: Comparison of Selective Index among breast cancer cell lines

Cells Lines	Selectivity index (SI) Mean ± SD
MCF-7	317.3 ± 65.4
	(230.61 - 455.97)
MDA-MB-231	250.69 ± 50.10
	(187.64 - 356.07)
P-value	0.007**

Mean ± SD, (Min - Max), **Significant at 1%

Table 6: Comparison of Different variables of Trypan blue dye exclusion assay among different dosages of metformin

Doses (μM)	Viable Cells Mean ± SD N=12	Total Cells Mean ± SD N=12	Viability (%) Mean ± SD N=12	Death cells Mean ± SD N=12
0	263.191±1.64	267.21±1.58	98.8 ± 0.3	4.017±0.287
	(261.80-265.00)	(266.10-269.02)	(98.58 - 99.17)	(3.726-4.300)
2.5	240.31±2.721	263.15±0.232	89.88±1.027	22.842±2.867
	(237.70-243.13)	(262.95-263.40)	(88.72-90.67)	(19.97-25.705)
5	211.52±2.17	260.30±4.25	81.03±0.334	48.781±2.100
	(209.17-213.46)	(255.87-264.36)	(80.75-81.40)	(46.700-50.9)
7.5	186.62±5.07	258.08±5.72	71.98±0.504	71.464±1.034
	(180.92-190.68)	(251.97-263.32)	(71.42-72.40)	(70.701-72.64)
10.5	160.43±6.22	255.71±7.36	62.17±1.56	95.281±1.188
	(154.0-166.42)	(247.92-262.56)	(60.42-63.45)	(93.925-96.140)
12	132.95±6.9	253.20±8.71	52.3 ± 1.9	120.253±2.628
	(126.6-140.3)	(243.95-261.24)	(50.27 - 53.9)	(117.35-122.469)
15	101.198±6.176	253.01±7.58	40.2 ± 2.8	151.816±6.415
	(100.28-295.53)	(245.30-260.46)	(37.27 - 42.87)	(145.014-157.757)
P-value	0.003**	0.05*	0.003**	0.003**

N=4 samples per day for each dose for 3 days so N=12 but Data analysis were done after entering mean value for each dose for each day so N became 4 for each dose and Total N=21 for individual drug
'Mean ± SD in x 10^4
'(Min - Max) in x 10^4
**Significant at 1%; *Significant at 5%

manner with average percentage decrease and IC50 - 60.125 and 1.6±0.1 respectively these findings were compatible with study led by Liu *et al*. (2015). As they perceive the prospective anticancerous action of Phenformin on various breast tumor cells; they acknowledged that Phenformin ominously reduces the viabilities of all studied breast cancerous cell lines and IC50 were 2.347±0.010, 1.885±0.015 and 1.184±0.045 for MDA-MB-231, SUM1315, MCF7 cell lines separately.

Correspondingly for MDA-MB-231 dose dependent cytotoxic effects of Metformin established by dwindled the percentage viability with average percentage decrease and IC50 were -48.12 and 2.3±0.5, this was in harmony to trial directed by Hadad *et al*. (2014). They studied the impacts of metformin on breast cancerous cell lines (MDA-MB-231 plus MCF-7) to determine vital system of Metformin by which cell development was constrained in these cells. They reasoned that fundamental system through which metformin diminishes the proliferation in these cancerous cells via influencing the vitality sensor pathway recognized as AMPK pathway.

Dose dependent impact of metformin on MCF-7 as assessed by means of Trypan blue dye exclusion assay uncovered that Metformin honorably reduces the viable cell count (χ^2 (2) =19.636, p=0.003) and percentage feasibility (χ^2 (2) =19.636, p=0.003), these outcomes were in accordance with the examination directed by Queiroz *et al*. (2014). They evaluated the impending antiproliferating action of metformin in MCF-7 cell line. They revealed that contrasted with control metformin successfully diminishes the feasibility rate surveyed by means of Trypan blue dye exclusion and MTT assays.

Metformin potentially captures the cancer cells progression at stage of G0-G1 and expands the programmed cell death conceivably by means of accelerations of AMPK pathway , increase expression of FOXO and p27 and induction of apoptotic protein, for example, Bcl-2 and reductions of action of IRβ, Akt and ERK1/2 (Hadad *et al*., 2014).

In a perfect world anticancerous medication ought not to disturb viability of normal epithelial cells as appeared in our investigation that for MCF-10 metformin is unequipped for diminishing the proliferation of MCF-10 cell line, these findings were in consistence with Safari *et al*. (2015). They assessed the antiproliferative action of metformin on malignant cells (MCF-7) and non-malignant cell culture (HEK293) in both lower oxygen and normal oxygen circumstances. They reasoned that metformin was not able reduction the multiplication of non-cancerous cells in equally hypoxic (lower oxygen) and normoxic (normal oxygen) situations, while essentially wane the rate feasibility of malignant cells (MCF-7) under predominantly hypoxic conditions.

Another examination led by Hirsch *et al*. (2009) demonstrated that metformin specifically focus on the growth undifferentiated cells of breast tumor notwithstanding their cytotoxic impacts on breast cancerous cell lines. To show the defensive impacts of metformin, they utilized MCF10A, MCF7, MDA-MB-468 and SKBR3. They concluded that metformin

significantly reduces the viabilities of cancerous cells of breast without any deleterious effects on normal breast cells.

Limitations of Study: Because of some laboratory issues the triplicate could not performed on same batch of cancerous cell lines. So to avoid bias we taken statistical analytical test that applicable for independent variables.

CONCLUSION

In exploration of effective oral cost-effective adjuvant anticancerous drugs this study revealed that metformin can meritoriously reduce the viability of cancerous cells without any deleterious effects on normal epithelial cells.

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