Print ISSN: 2319-2003 | Online ISSN: 2279-0780

IJBCP International Journal of Basic & Clinical Pharmacology

DOI: http://dx.doi.org/10.18203/2319-2003.ijbcp20182669

Original Research Article

Proliferative effect of the calcium channel blocker Nifedipine on human embryonic kidney cells

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Received: 10 October 2017
Accepted: 01 November 2017

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ABSTRACT

Background: Numerous epidemiological studies have shown a positive as well as negative association between chronic use of calcium channel blockers and the increased risk of developing cancer. However, these associations were enmeshed with controversies in the absence of laboratory based studies to back up those claims. The aim was to determine in mechanistic terms the association between the long-term administrations of nifedipineand increased risk of developing cancer with the aid of human embryonic kidney (HEK293) cell line.
Methods: Cell counting using the Trypan blue dye exclusion and 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) assays were used to investigate the effect of nifedipine on the growth pattern of HEK293 cells.
Results: Nifedipine had a proliferative effect on HEK293 cells growth and this proliferation is more profound at low concentrations of nifedipine than high concentrations and the proliferation was statistically significant ($p<0.01$).
Conclusions: The chronic use of nifedipine is associated with increased proliferation of cells with concomitant elevation of polyamines concentration and elevated polyamine levels have been implicated in many malignant transformations and hence, these provide possible explanation on the link between long term use of nifedipine and development of some human cancers.

Keywords: Antihypertensive agents, Cancer, Growth inhibition, Nifedipine, Polyamines, Proliferation

INTRODUCTION

Shortly after the first study linking the use of reserpine and increased risk of cancer development in the last three decades, several reports have been put forward linking the use of different classes and types of antihypertensive agents to cancer development. However, these reports have been trailed by uncertainties with some suggesting

positive, negative or no associations. Examples of such association is that of use of beta-blockers and lung cancer development, calcium channel blockers (CCBs) and general cancers while thiazide diuretics had increased risk of kidney and colon cancers. Furthermore, antihypertensive medicines were found to be associated with increased risk of developing glioma.[1]

Polyamines have been found to play a crucial role in cancer pathogenesis with their elevation seen in significant number of human epithelial tumours and this is due to alteration of genes, expression levels and enzymes activities in the polyamines metabolic pathway.[2] The polyamines putrescine, spermidine and spermine play crucial roles in the normal proliferation and differentiation of cells and are markedly elevated in malignant transformation.[3] The polyamines are omnipresent in nature and are regulated by the biosynthetic enzymes which includes ornithine decarboxylase (ODC).[4] Consequently, the pathways for polyamine biosynthesis, degradation and transport are currently explored for cancer chemoprevention and chemotherapy.[5-7]

Link between calcium channel blockers (CCBs) and cancer development

Nifedipine and nicardipine being CCBs were discovered to induce gingival overgrowth among patients being treated with these medicines. In fact, they were found to induce an increase in DNA and type-1 collagen synthesis and enhanced proliferation rate of cells.[8] Increased ductal and lobular breast cancer risk was discovered following long term use of CCB for greater than 10 years in women aged 55 – 74 years in the US.[9]

Similarly, CCB was found not to enhance the growth of tumour cells in several experimental models.[10] On the contrary, inhibits the growth of tumour cells in certain neoplasia models and effectively served as adjuvant therapy in specific drug resistant cancer cells. Nifedipine was found to significantly increase the antitumor action of cisplatin against glioblastoma-1 (GB-1) cells.[11] Nifedipine increased cisplatin cytotoxicity in the absence of normal extracellular calcium ion. Furthermore, the synergistic anti-tumour effect of cisplatin and nifedipine was truncated by actinomycin D and cycloheximide, indicating reliance of such effect on synthesis of new RNA and protein. The non-cytotoxic nifedipine was confirmed to improved anti-tumour activity of cisplatin via a synergistic fashion on multidrug-resistant GB-1 cells devoid of calcium dependent endonuclease, which subsequently leads to apoptosis through interaction with another unidentified functional site apart from the calcium channel on GB-1 cells. The CCB flunarizine was found to be cytotoxic to Jurkat T-leukaemia cells and other haematological malignancies but not toxic to breast or colon cancer cells. Flunarizine treatment was associated with the activation of caspase-3, poly (ADP-ribose) polymerase cleavage and DNA fragments laddering, all these represent the hallmark of apoptosis.[12]

Furthermore, evidence linking CCB to increased risk of cancer is lacking despite comprehensive epidemiological studies which were conducted to hitherto prove an association.[10] There was no link between CCB use and overall cancer risk and its use was not significantly linked with increased risk of cancers individually, in addition to the previously implicated cancers except for kidney cancer. Patients who were exposed to CCBs were compared with two cohorts for comparison, that is, matched patients not exposed to CCB (non-CCB) and unmatched patients not exposed to CCB and at least any other antihypertensive (AHT) medication. Following analysis based on the duration of exposure to CCB, excess risk to cancer was not observed.[13]

Aim of the study was to evaluate in mechanistic term, the association between chronic use of nifedipine and cancer development using HEK293 cell line.

Specific objectives

- Evaluate the proliferative effect of nifedipine on HEK293 cells.
- Determine the polyamine content of HEK293 cells treated with nifedipine.

METHODS

Reagents

Minimum Essential eagle Medium (EMEM), Penicillin, Streptomycin, L-glutamine, Dimethyl Sulfoxide (DMSO), sterile ethanol, nifedipine, fetal bovine serum (FBS), phosphate buffered saline (PBS), sodium chloride, trypsin and the human embryonic kidney (HEK293) cell line obtained from ECACC.

Nifedipine preparation

Distilled water was used to dissolved nifedipine and subsequently different concentrations of the drug were made using the prepared culture medium.

Cell line

Human Embryonic Kidney (HEK) 293 cell line was used. The cells grow easily in tissue culture and can be easily transfected as well. They are immortalized human embryonic kidney cells which were obtained from ECACC and derived from epithelium and SV40 transformed cells from a primary culture of fetal kidney. They possess some unusual characteristics of having features of neuronal cells.[14] The cells were maintained in Minimum Essential Medium, alpha modification with 2 mM L-glutamine, 10% (v/v) foetal bovine serum (FBS), 5000 µg/ml Penicillin and 5000U/ml Streptomycin. Cells were passed routinely every 3-4 days at a confluence with a split ratio of 1:3 to 1:5. Phase contrast microscopy was used to observe the monolayer and confluence determination. Cells were used for about 15 passages.

MTT assay

The aim of 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) assay is to access metabolic activity of cells. The MTT assay is dependent on the conversion of yellow water soluble MTT into some insoluble formazan purple crystals by living cells which eventually denotes activity of the mitochondria. MTT assay was performed on 96 well sterile cell culture plates. The flat-bottomed plates were used.

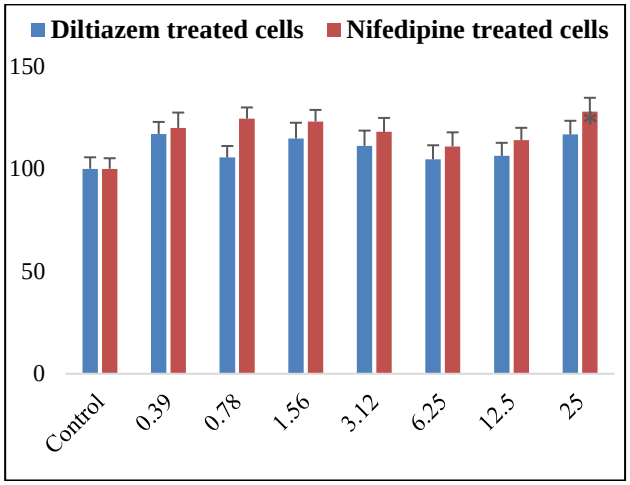
Statistical analysis

Statistical analysis was performed from three independent experiments carried out to ascertain the effect of nifedipine on the viability of HEK293 cells. Analysis of variance (ANOVA) with Dunnet's post-test was applied for multiple drug exposures and results expressed as mean ± standard error of the mean (SEM). The Statistical Package for Social Science (SPSS) version 21 and Microsoft excel 2016 was used for statistical analysis and plotting of graph respectively.

RESULTS

Effect of high concentrations of diltiazem and nifedipine on HEK293 cell line

Using the MTT assay the extent of the effect of high concentrations of diltiazem and nifedipine were determined (Figure 1). The concentrations used were 0.39, 0.78, 1.56, 3.13, 6.25, 12.5 and 25µM.



(n = 3 with 6 replicates per experiment)

Figure 1: Effect of high concentration of diltiazem and nifedipine on HEK293 cells.

Both drugs were observed to have caused increased proliferation of the cells with more proliferations observed with nifedipine at 0.78 and 25µM. The difference in proliferation between the control and drug treated cells was found to be significant ($p<0.05$) (Figure 1).

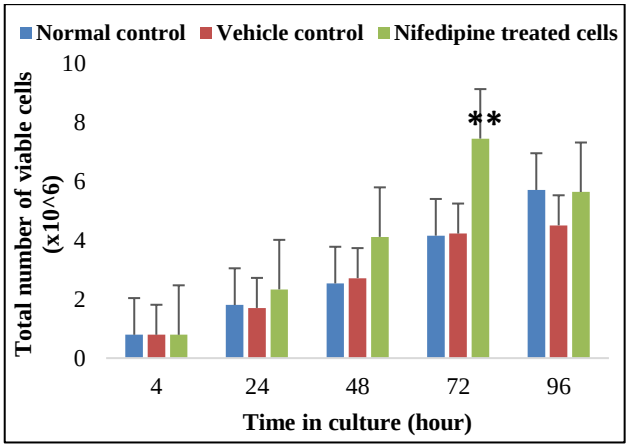
The effect of 1µM nifedipine on cell number and viability

Nifedipine had proliferative effect on HEK293 cells (Figure 2). The viability of the cells remained above 97% in the majority of the experiments (Table 1). However, nifedipine caused a prolonged exponential phase of the cell growth. The cell proliferation was profound at the 72-hour time point in culture and this proliferative effect was found to be significant ($p<0.01$) when compared with control (Figure 2).

Table 1: Viability of HEK293 cells treated with 1µM of nifedipine.

Time (hour)	Normal control (%)	Vehicle control (%)	Nifedipine treated cells (%)
24	98.90	97.98	99.47
48	98.25	98.77	98.21
72	98.34	98.25	97.54
96	83.80	90.91	98.46

(n = 3 with 2 replicates per experiment)



(n = 3 with 2 replicates per time point per experiment)

Figure 2: Growth of HEK293 cells overtime with 1 µM of nifedipine treatment.

Effect of 48 and 72 h exposure to 1µM of nifedipine on intracellular polyamine content

The measured polyamine content of the cultured cells treated with 1µM of nifedipine showed an increase in both total and individual polyamine content at both 48 and 72 h exposure time point when compared with control cells (Table 2).

HEK293 cells were seeded at a density of 2.4 x 10^4cells/cm^2 on 96 well microtitre plates and allowed to attach and grow for 48 hours. After 48 hours the medium was replaced with medium containing drug or vehicle. Plates were incubated for 48 hours and assayed based on the protocol of the MTT assay. Values are mean±SD. * P <0.05 one-way ANOVA with Dunnett's post-test.

Cells were seeded at a density of 2.4 x 10^4cells/cm^2 on 5cm diameter tissue culture plates in duplicate. The cells were allowed to attach for a period of 4 hours at which point the first plates were harvested - time 0. Plates were harvested using the trypsin/EDTA method and counted using a haemocytometer. The polyamine fraction and protein were collected. Values are mean±S.E.M. ** P <0.01 one-way ANOVA with Dunnett's post-test.

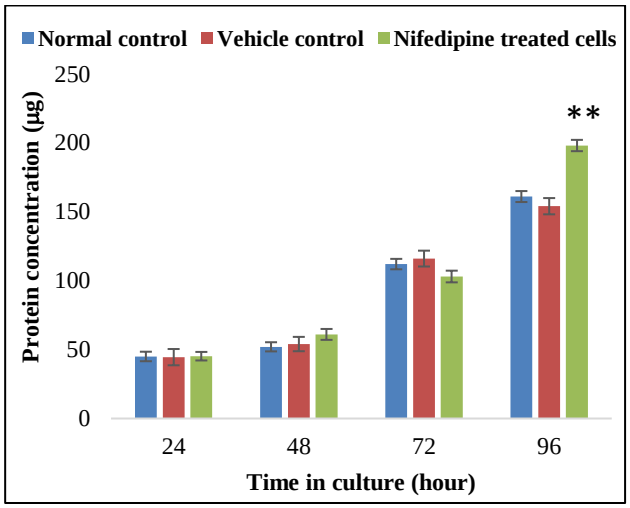
Table 2: Polyamine content of HEK293 cells.

Time in culture (h)	Treatment	Putrescine (nmol/µg protein)	Spermidine (nmol/µg protein)	Spermine (nmol/µg protein)	Total (nmol/µg protein)
48	Control	0.18±0.03	1.43±0.15	4.19±0.38	5.8±0.56
	Nifedipine	0.31±0.03	1.91±0.12	5.53±0.04	7.75±0.19
72	Control	0.66±0.06	4.03±0.20	8.73±0.38	13.42±0.64
	Nifedipine	0.84±0.14	4.41±0.75	11.68±2.10	16.93±2.99

Cells were seeded at a density of 2.4 x 10^4cells/cm^2 on 5cm diameter tissue culture plates in duplicate. The cells were allowed to attach for a period of 4 hours at which point the first plates were harvested - time 0. Cells were harvested using the trypsin/EDTA method every 24 hour and counted using an improved Neubauer haemocytometer. The viability was determined from total cell number and total viable cell number. Not statistically significant, analysed using two-way ANOVA.

Protein content of HEK293 cells treated with 25µM of nifedipine

The measured total protein content of the cultured cells treated with 25µM of nifedipine showed increased total protein content with increasing time of the cells in culture, which was shown in the pattern of growth of the cells and the increase in protein content follows a sigmoid pattern (Figure 3). The increased in the protein content was found to be significant (p<0.01).



(n = 3 with 2 replicates per time point per experiment).

Figure 3: Protein content of HEK293 cells treated with 25µM of nifedipine.

Cells were seeded on 5cm diameter tissue culture dishes at a density of 2.4 x 10^4cells/cm^2 and allowed to attach for 4 hours. Cells were then subsequently harvested every 24 hours. Protein was determined based on the modified Lowry method. Results expressed as mean protein concentration (µg)±S.E.M. ** P <0.01 one-way ANOVA with Dunnett's post-test.

Cells were seeded at a density of 2.4 x 10^4cells/cm^2 on 5 cm diameter tissue culture plates in duplicate. The cells were allowed to attach and grow for a period of 24 hours prior to the medium being removed. The medium was replaced with fresh pre-warmed medium containing 1µM of nifedipine. Cells were exposed for a period of 48 and 72h before they were harvested respectively. Polyamine fraction was analysed by pre-column derivatization method and the fluorescent tag used was dansyl chloride which adhere to the secondary polyamine groups within the polyamine structure and expressed per µg of protein using the Lowry method for protein determination. n = 3±S.E.M.

DISCUSSION

The hypothesis of this study was that there is a link between chronic use of nifedipine and increased risk of cancer development using human embryonic kidney (HEK293) cell line.

It was postulated that this will occur via some number of mechanisms which are increased proliferation of cells and increased intracellular polyamine concentrations.

Following repeated treatment of the cells with different concentrations of nifedipine, we observed an increase in the number of cells relative to the control cells using the trypan blue exclusion assay and the effect was seen commonly with long term exposure of the cells to nifedipine indicating chronic exposure is required to trigger increase proliferation of the cells.

Similarly, authors discovered using the MTT assay, a significant increased proliferation rate of the HEK293 cell line when it was incubated with various concentrations of diltiazem and nifedipine. Interestingly, the increased proliferation rate was more profound at lowest and highest concentration values. Our findings supported a previous work where it was discovered that nifedipine and nicardipine increased proliferation of cells leading to hyperplasia and gingival overgrowth.[8,15,16]

The increase in cell proliferation induced by nifedipine and diltiazem may explain the results of the many epidemiological studies linking long term use of CCBs to increased risk of cancer and such studies included those that found patients treated with CCBs (nifedipine, diltiazem and verapamil) for a long term having increased risk of developing cancer with relative risk of 2.02 and 95% confidence interval of 1.16-3.54.[17] In the same token, a similar study found increased ductal and lobular breast cancer risk in women aged 55-74 years in the US who were treated with CCBs for greater than ten years.[9] Similarly, increased risk of cancer was found with the use of CCBs for more than thirteen years with odds ratio of 1.09 and 95% confidence interval of 1.03-1.16.[18]

Contrary to the above findings, some epidemiological studies did not find any link between long term use of CCBs and increased risk of cancer.[19,10,13] Also, contrary to our findings, nifedipine inhibited the growth of tumor cells in certain neoplasia models and effectively served as adjuvant therapy in specific drug resistant cancer cells treatment.[10]

The protein as well as the polyamine content of the cell line treated with nifedipine was found to be significantly increased as against the control cells. The significantly increased protein content of the nifedipine treated cells reflected the increased proliferation of the cells**.** Likewise, polyamines both total and individuals including putrescine, spermidine and spermine were all significantly elevated following treatment of the cells with nifedipine. Physiologically, the intracellular concentrations of polyamines are tightly and strictly regulated to remain within normal physiological range but its elevation in these cells indicate an ongoing malignant transformation based on the role of increased level of polyamines in cancerous cells influenced by high level of ornithine decarboxylase enzyme.[20,21] The increased polyamines level is indicative of dysregulated biosynthetic, bio-degradative and transport pathways which are hallmarks of cancer pathogenesis. The role of elevated levels of polyamines in cancer have been well research and documented.[22-25]

CONCLUSION

Some calcium channel blockers and nifedipine do have profound proliferative effect on HEK293 cell lines. The proliferation resulted in increased level of proteins and polyamine contents: elevated levels of polyamines have been implicated in many malignant transformations. Therefore, there is a possible association between chronic use of nifedipine and increased risk of developing cancer.

ACKNOWLEDGEMENTS

Authors would like to give deepest gratitude and appreciation goes to their employers for sponsoring the research. Also, special gratitude to all their teachers and Professors for their steadfast mentoring and stewardship.

Funding: No funding sources
Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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Cite this article as: Ahmad JL, Okebaram CC, Ogbuagu EO, Ogbonnaya CN, Asobie GC. Proliferative effect of the calcium channel blocker Nifedipine on human embryonic kidney cells. Int J Basic Clin Pharmacol 2018;7:1226-31.