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Research Paper

Repurposing of sildenafil as antitumour; induction of cyclic guanosine monophosphate/protein kinase G pathway, caspase-dependent apoptosis and pivotal reduction of Nuclear factor kappa light chain enhancer of activated B cells in lung cancer

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Abstract

Objectives Lung cancer is one of the most frequent types of cancers that lead to death. Sildenafil is a potent inhibitor of phosphodiesterase-5 and showed potential anticancer effects, which has not yet been fully evaluated. Thus, this study aims to investigate the potential anticancer effect of sildenafil in urethane-induced lung cancer in BALB/c mice.

Methods Five-week-old male BALB/c mice were treated with either (i) normal saline only, (ii) sildenafil only 50 mg kg-1/ P.O every other day for the last four successive weeks, (iii) urethane 1.5 gm kg-1 i.p (at day 1 and day 60), (iv) carboplatin after urethane induction, or (v) sildenafil after urethane induction.

Key findings It was shown that sildenafil significantly increased the levels of cGMP and Caspase-3 with a reduction of NF-κB, Bcl-2, Cyclin D1, intercellular adhesion molecule 1, matrix metalloproteinase-2 levels and normalisation of Nrf2 along with pronounced improvement in the histological patterns.

Conclusions These results indicated that sildenafil markedly induces cell cycle arrest, apoptosis and inhibits the metastatic activity through activation of cyclic guanosine monophosphate/protein kinase G pathway and down-regulation of cyclin D1 and nuclear factor kappa light chain enhancer of activated B cells with downstream anti-apoptotic gene Bcl-2, which underscores the critical importance of future using sildenafil in the treatment of lung cancer.

Key words: urethane; lung cancer; sildenafil; apoptosis; PDE5

Introduction

Among different types of cancer, lung cancer is one of the most common causes of death, in addition to 1.8 million new cases has been estimated with 12.7% of all types of new cancer cases.[1] It has become the common cause of cancer death among males and the second (after breast cancer) in females, representing 19% of all cancers' death.[2] According to the World Health Organization, lung cancer is mainly classified into small cell lung cancer (SCLC) and a non-small cell lung cancer

(NSCLC).[3] NSCLC is the dominant type with 80% of lung cancer cases and subclassified into several subclasses such as adeno, squamous cell, adenosquamous cell, large cell and sarcomatoid carcinomas.[4] In Egypt, lung cancer has been recorded as one of the top-ranked five types of cancer (liver, urinary bladder, breast and brain) with adenocarcinoma as the most frequent subtype of all lung cancer cases.[5,6]

Treatment strategies of lung cancer may include surgery, chemotherapy, radiotherapy, targeted therapy and recently immunotherapy depending on the stage and the type of lung cancer.[7] Platinum-based drugs (cisplatin or carboplatin) are the main treatment and used as a first-line therapy for advanced NSCLC.[8] By virtue of the relapse in most cases, resistance and severe adverse effects of platinum-based drugs that are commonly encountered among patients with lung cancer[9] consequently, great interest is argued to develop other treatment modalities such as targeted and immunotherapy were developed.[10] Unfortunately, resistance from targeted therapy and immunotherapy-associated autoimmune disorders are life-threatening conditions.[7] Moreover, lung cancer usually diagnosed at a late stage of progression therefore there is an urgent need for the development of novel strategic modalities for lung cancer treatment.[11] Using off-label established drugs as anticancer drugs, such as statins,[12] metformin,[13] β-blockers[14] as well as phosphodiesterase-5 (PDE5) inhibitors,[15] is a new strategy for treating cancer.

Interestingly, overexpression of PDE5 is detected in several types of cancers including colon, prostate, breast, pancreatic and NSCLC.[16] In addition, it has been reported that the increase in cGMP and activation of protein kinase G (PKG) enhances apoptosis caspase pathway and inhibits cell growth of colorectal and prostate cancer cells.[17, 18] Accordingly, PDE5 plays an essential role in carcinogenesis thereby targeting PDE5, which may be a potential target for cancer prevention and treatment.

Sildenafil is a potent selective inhibitor of PDE5 and is widely used for the treatment of erectile dysfunction and pulmonary hypertension.[19] It binds to the catalytic site of the PDE5 enzyme the preventing breakdown of cGMP enhancing cGMP binding to the regulatory domain, which in turn potentiates the catalytic binding of sildenafil.[20] Then, cGMP activates PKG resulted in phosphorylation of many proteins for Ca^{+2} hemostasis followed by lowering intracellular Ca^{+2} and consequent smooth muscle relaxation.[21]

Although, cytotoxicity of sildenafil as monotherapy or chemoadjuvant therapy, besides its sensitising effect of chemotherapeutics, has been well documented in previous literature in a wide variety of cancer cells inducing apoptosis both in vitro and in vivo, its mechanistic role in lung cancer warrants further investigations, especially because of its potent effect in other pulmonary disorders.[22] In addition, sildenafil may have an additive benefit through improving outcomes in patients with pulmonary hypertension and pulmonary oedema as a consequence of lung cancer itself.[23] Moreover, increasing drug targeting to tumour cells is a valuable approach that can be achieved with sildenafil as it increases blood flow to PDE5 rich vasculatures (as lung arterial vessels) by several folds that may improve pulmonary drug distribution.[24] Consequently, sildenafil may be a precious candidate for lung cancer chemoadjuvant therapy especially as it has a well-established pharmacokinetic profile.[25,26]

This study seeks to shed a light on the possible mechanistic pathway of sildenafil anticancer activity in urethane-induced NSCLC in BALB/c mice.

Materials and Methods

Animals

Five-week-old male BALB/c mice (22–30 g) were purchased from Animal House Colony, Pharmacology and Chemistry Research Center, Misr University and Technology Park, 6 October City, Egypt. Mice were housed in polycarbonate cages at the Animal House Lab., Faculty of Pharmacy, Beni-Suef University. All animals were kept in a controlled room at temperature (25 ± 1°C) and humidity (55–70%) with 12-h light/dark cycles and were maintained on a standard lab diet and water ad libitum. Before the experiment, the mice were allowed for one week of acclimatisation. All animal care and experimental procedures were performed in accordance with the guidelines of the institutional animal care and use committee (IACUC) of Beni-Suef University following recommendations of the Guide for Care and Use of Laboratory Animals (National Institutes of Health, publication no. 85–23, revised 1985). Animal studies are reported in compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.[27, 28]

Induction of lung cancer

Lung cancer was induced according to previous work as described by Hamzawy *et al*.[29] Urethane was intraperitoneal injected at dose 1.5 gm/kg, on the first day of the study period and the second dose was injected after 2 months.[29]

Experimental design

After one week of acclimatisation, 75 weight-matched healthy BALB/c mice were randomly assigned into five groups (15 mice/group) as follows for 5 months:

Group I

Mice treated with normal saline only (normal control group).

Group II

Mice treated with sildenafil only 50 mg kg^{-1}/P.O every other day for the last four successive weeks.[30]

Group III

Mice treated with urethane 1.5 g kg^{-1} i.p (at day one and day 60).

Group IV

Mice treated with carboplatin after urethane induction at a dose of 15 mg kg^{-1} i.p every other day for the last four successive weeks.[31]

Group V

Mice received sildenafil orally after urethane induction at a dose of 50 mg kg^{-1} every other day for the last four successive weeks.

At the end of the experimental period (i.e., day 150), blood samples were collected from each mouse from retro-orbital vein under ketamine (12.5 mg kg^{-1}) and xylazine (1.5 mg kg^{-1}) anaesthesia[32] using non-heparinised microhematocrite capillary tubes and allowed to clot at room temperature, then for serum separation centrifugation was done at 3000 × *g* for 20 min, the sera were stored at –20°C for determination of lung cancer biomarkers; cytokeratin-19 fragments (CYFRA21-1) and Insulin-like growth factor-1(IGF-1).

Thereafter the collection of blood samples, mice were sacrificed by decapitation under light anaesthesia and their lungs were sampled. Lung tumour volumes were calculated using a calliper. Then, each lung was divided into three parts; one part was fixed in 10% formalin for histopathological examinations, the second part for western blotting assay of nuclear factor kappa light chain enhancer of activated B cells (NF-κB), caspase-3 and nuclear factor erythroid-2 related factor-2 (Nrf2) and the remaining part was weighed to be 0.05–0.1 g for homogenisation at phosphate buffer (pH 7.4) to obtain 20% w/v lung homogenate. The homogenate was then

centrifuged by cooling centrifuge (4°C) at 1700 × *g* for 10 min. The supernatant was stored at −80°C for further assessment of cyclic guanosine monophosphate (cGMP), B-cell lymphoma 2 (Bcl-2), cyclin D1, matrix metalloproteinase-2 (MMP-2) and intercellular adhesion molecule 1 (ICAM-1).

Calculation of lung tumour volumes

Lung tumour volumes were measured by calliper using the formula: volume (mm^3) = $(l \times w^2)/2$, while l (length) is the longest diameter and w (width) is the diameter perpendicular to the length as previously prescribed.[33,34]

Determination of lung tumour biomarkers

CYFRA 21-1 and IGF-1 were measured in the serum from all treated groups using the enzyme-linked immunosorbent assay (ELIZA) kits according to the manufacturer's instructions.[35,36]

Determination of cGMP lung content

cGMP content in lung tissues from each mouse was estimated using an ELISA kit.[37] Briefly, the samples (tissue homogenate), standards and controls were added to the plate wells then the plate was incubated for 90 min at 37°C. After the incubation, the plate was washed two times with the washing buffer then the biotinylated mouse cGMP antibody was added to each well and the plate was incubated for 60 min at 37°C then washed again with the washing buffer three times. The enzyme conjugate was added to each well except the blank wells then the plate was incubated for 30 min at 37°C and washed five times. The colouring reagent was added to each well then the plate was incubated in dark at 37°C. Finally, colouring reagent C was added and the O.D. was read at 450 nm within 30 min.

Determination of apoptotic activity and cell cycle progression

Assessment of the anti-apoptotic Bcl-2 and cyclin D1 contents in lung tissues was done using ELISA kits according to the manufacturer's instructions.

Determination of metastasis and angiogenesis biomarkers

MMP-2 and ICAM-1 biomarkers were detected in lung tissues using ELISA kits according to the methods that were previously published.[38,39]

Western blot analysis of NF-κB, Nrf2 and Caspase-3

The immunoblotting assay was previously described.[40] Lung tissues (50 mg per mouse) were rinsed two times with cold phosphate buffered saline (PBS) then homogenised with 1.5 ml cold radioimmunoprecipitation assay (RIPA) lysis buffer containing 150 mM NaCl, 1% NP-40 or 0.1% triton, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate) and 50 mM Tris–HCl (pH = 8). The lysate was centrifuged at 16 000 × *g* for 30 min at 4°C and the supernatant was collected and boiled at 95°C for 5 min to ensure the complete denaturation of proteins. The proteins were separated by SDS-PAGE gel (sodium dodecyl sulphate–poly acrylamide gel electrophoresis). The gel was submerged and a prepared running buffer of 190 mM glycine, 0.1% SDS, and 25 mM Tris-base (pH = 8.3) were poured into the electrophoresis chamber. After that, the gel was run for 20 min at 50 V then the voltage was increased to 100–150 V to allow protein migration and separation and finish the run in about 1 h. A transfer sandwich composed of filter paper, polyvinylidene fluoride (PVDF) membrane, the gel and filter paper (from below to above) was placed in the transfer tank with the transfer buffer (190 mM glycine, 20% methanol and 25 mM Tris-base with PH were adjusted to pH 8.3) then the blot was run for 7 min at 25 V to allow the transfer of protein bands from the gel to the membrane. tris-buffered saline with Tween 20 (TBST) and 3% bovine serum albumin (BSA) for 1 h blocked the membrane. The primary antibodies are specific for NF-kB, Nrf2 or activated Caspase-3 was diluted with TBST (1 : 2000) and incubated overnight at 4°C with the membrane then a horseradish peroxidase (HRP)-the conjugated secondary solution was incubated with the blotted proteins for 1 h at room temperature and the membrane was rinsed with TBST before and after the incubation.

Finally, the chemiluminescent substrate (BIO-RAD, Hercules, CA, USA) was applied according to the manufacturer's recommendation then the signals were captured using a CCD camera-based imager and the band intensity was of protein expression was quantified against the control sample after normalisation by beta-actin using Bio-Rad image analysis software. Each experiment was repeated at least three times.

Histopathological examination of lung

Isolated lung samples were fixed in 10% formalin for 48 h for histopathological examinations, then washed with water for 1 h. For dehydration, a serial dilution of ethyl alcohol (50, 70, 95 and 100%), 2 h each, were used. Samples were cleared in xylene (two changes, 2 h each) then embedded in paraffin wax in a hot air oven at 70°C (three changes, 2 h each). The blocks of the paraffin wax were sectioned a using sliding microtome (5 μm) then the sections were mounted on clean glass slides. Haematoxylin and Eosin stain (H&E) was used for staining. Three to four sections from each lung were examined. An electric light microscope was used to examine the stained sections and the photomicrographs were taken.[41]

Data and statistical analysis

The data and statistical analysis complied with the recommendations on experimental design and analysis in pharmacology.[42] The data were expressed as the mean ± SEM using the SPSS program (version 22).

One-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test was used to compare the means of different groups.[43] The level of significance was $p < 0.05$.

Materials

Urethane was obtained from Sigma Chemical Company, St. Louis, USA. Carboplatin was purchased from the local market as carboplatin 150 mg/15 ml vial (Carboplatin, Mylan, France) and sildenafil was obtained from National Organization for Drug Control and Research (NODCAR), Egypt. Ketamine and xylazine were obtained as ketamine 50 mg/ml vial (SIGMA TEC Pharmaceutical industries-Egypt) and Xyla-ject 20 mg/ml vial (ADWIA Co. S. A. E. 10 of Ramadan city, Cairo, Egypt), respectively. Ethyl alcohol, xylene, paraffin wax and 10% formalin were procured from El Nasr Pharmaceutical Company, Egypt. IGF-1 reagent kit was obtained from Alpco Diagnostics, Salem, NH while CYFRA21-1 reagent kit was supplied from Genorise Scientific, Inc., USA. cGMP, Bcl-2, Cyclin D1, MMP-2 and ICAM-1 reagent kits were purchased from MyBioSource, USA. NF-κB, Nrf2 and Caspase-3 primary antibodies

for western blot were obtained from Thermo Fisher Scientific, USA. An HRP-the conjugated secondary solution was purchased from Novus Biologicals (Littleton, Colorado, USA). All other chemicals and reagents used were obtained from certificate sources with high analytical grade.

Results

Effect of sildenafil on tumour volume, incidence and multiplicity in BALB/c mice treated with urethane-induced lung cancer.

As showed in Figure 1A and B tumour volume was significantly increased in animals treated with urethane in comparison to a normal control group. Animals treated with sildenafil showed a significant reduction of tumour volume after urethane treatment.

Figure 1C represented that tumours had been found in almost 80% (n = 15) of the mice treated with urethane at the end of the treatment period, while the tumour incidence (the number of mice with tumour) was significantly decreased to about 26.67 and 33.33% in carboplatin and sildenafil pre-treated groups with urethane, respectively.

The numbers of tumours per mouse have been graphically illustrated in Figure 1D that showed mice treated with urethane had about 22.4 ± 2.23 tumour per mouse. Sildenafil treatment succeeded to inhibit tumour multiplicity by 66.07%, while the carboplatin treated group exhibited 75.45% inhibition of multiplicity.

Effect of sildenafil on the bodyweight of BALB/c mice treated with urethane-induced lung cancer

As presented in Table 1, urethane treatment showed a significant reduction in body weight of BALB/c mice with a remarkable reduction by about 8.6%. Interestingly, treatment of urethane pre-treated groups with either carboplatin or sildenafil notably attenuated the percentage of bodyweight reduction to about 2.60 and 1.74%, respectively.

Results represented that sildenafil treatment was accompanied by about 7.08% weight gain more or less like control itself. Effect of sildenafil on serum level of IGF-1 and CYFRA 21-1 in urethane-induced lung cancer model in BALB/c mice.

The normal control values for serum level of IGF-1 and CYFRA 21-1 were 55.43 ± 1.44 pg ml^{-1} and 34.67 ± 1.47 pg ml^{-1}, respectively. The urethane-treated group showed strikingly increased serum level of IGF-1 and CYFRA 21-1 in comparison to values from control and sildenafil treated rats. Treatment with carboplatin or sildenafil after urethane showed significant attenuation of serum IGF-1 and CYFRA 21-1 level as shown in Figure 2A and B.

Effect of sildenafil on lung cGMP content in urethane-induced lung cancer model in BALB/c mice

As shown in Table 2, animals treated with sildenafil only showed a significant increase in lung tissue content of cGMP higher than that of the normal control group. Urethane treatment significantly restrained the content of cGMP in lung tissues. Sildenafil succeeded to restore cGMP activity in lung homogenate of pre-treated animals with urethane. In the same context, treatment with carboplatin after urethane induction significantly induced cGMP content.

Effect of sildenafil on lung apoptotic biomarkers and cell cycle progression; Bcl-2 and cyclin D1 in urethane-induced lung cancer model in BALB/c mice.

As shown in Figure 3A and B, the normal control values for the lung tissue content of Bcl-2 and cyclin D1 were 3.42 ± 0.34 ng ml^{-1} and 35.43 ± 1.5 ng ml^{-1}, respectively. Urethane treatment appreciably

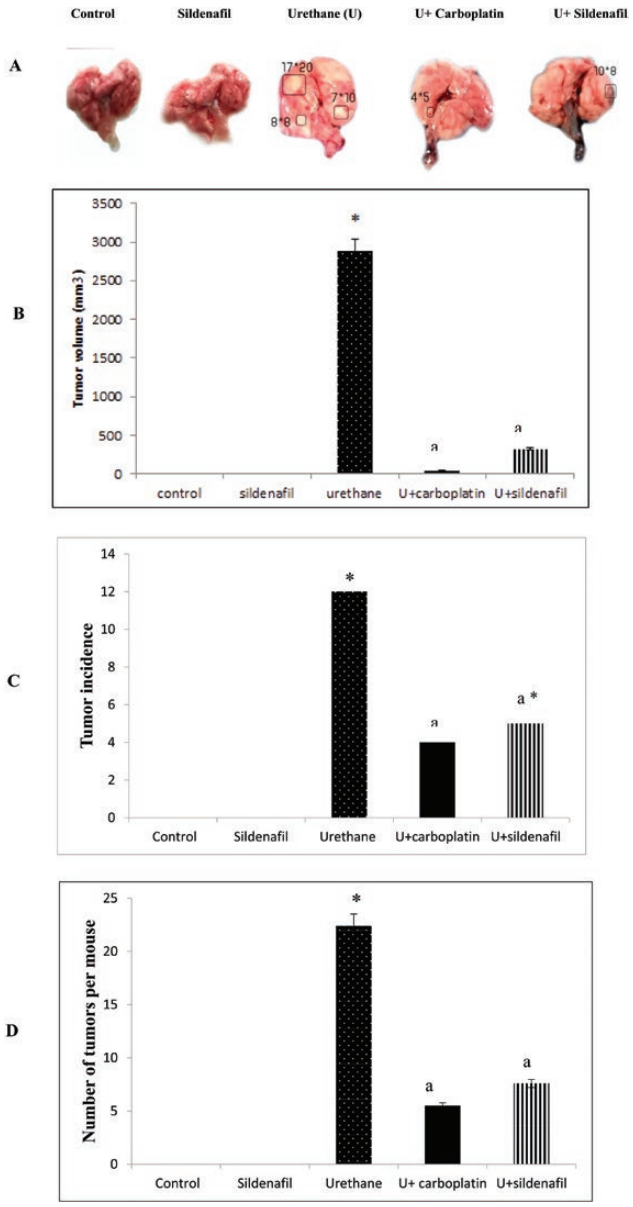

Figure 1 Effect of sildenafil on tumour volume, incidence and multiplicity in BALB/c mice treated with urethane-induced lung cancer. Data were expressed as mean ± SEM; n = 15 mice per group. One-way ANOVA followed by Tukey-Kramer multiple comparisons test was used to compare means of different groups. *Significantly different from normal control at $p < 0.05$. [a]Significantly different from urethane at $p < 0.05$.

raised the lung tissue content of Bcl-2 and cyclin D1 as compared to normal control. Animals that received sildenafil treatment after urethane-induced lung cancer showed a significant decline of Bcl-2 and cyclin D1, in the same manner with chemotherapy treatment carboplatin.

Effect of sildenafil on invasion biomarkers as ICAM-1 and MMP-2 in urethane-induced lung cancer in BALB/c mice.

The effects of sildenafil and carboplatin on the lung ICAM-1 and MMP-2 contents are depicted in Figure 4A and B. The results revealed that the normal control values for the lung tissue contents of ICAM-1 and MMP-2 were 1.9 ± 0.06 pg ml^{-1} and 1.15 ± 0.1 ng ml^{-1}, respectively. Urethane treatment obviously enhanced the lung tissue contents of ICAM-1 and MMP-2 as compared to normal control and sildenafil groups. Treatment with either sildenafil or carboplatin

Table 1 Effect of sildenafil on the weight of BALB/c mice treated with urethane-induced lung cancer

Weight (g)	Groups				
	Normal control	Sildenafil	Urethane	Urethane + carboplatin	Urethane + sildenafil
Before treatments (at the end of induction period, 120 days)	35.00 ± 0.21	34.80 ± 0.25	32.50 ± 0.06*	33.02 ± 0.11*	32.10 ± 0.09*
At the end of treatments period (30 days)	37.88 ± 0.62	37.42 ± 0.12	29.70 ± 0.15*	32.16 ± 0.23*[a]	31.54 ± 0.22*[a]
% of weight gain after treatment	**7.60%**	**7.08%**	–	–	–
% of weight inhibition after treatment	–	–	**8.6%**	**2.60%**	**1.74%**

Data were expressed as mean ± SEM; n = 15 mice per group. One-way ANOVA followed by Tukey-karmer multiple comparisons test was used to compare means of different groups.

Bold values indicate % of weight gain or % of wight inhibition of animal body weight in different treatment.

*Significantly different from normal control at $p < 0.05$.

[a]Significantly different from urethane at $p < 0.05$.

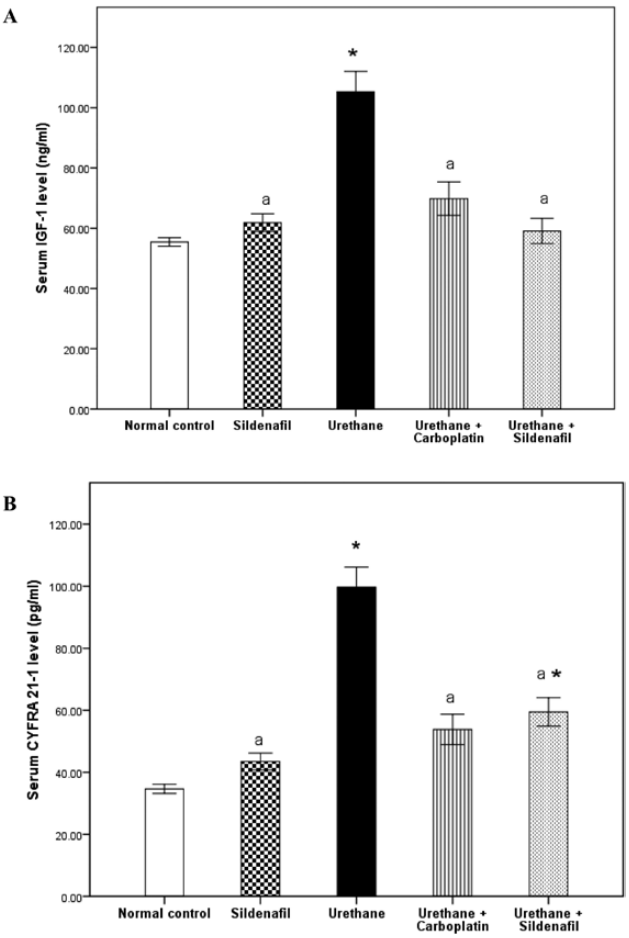

Figure 2 Effect of sildenafil on tumour markers as IGF-1 and CYFRA 21-1. (A) Effect of sildenafil on serum level of insulin-like growth factor-1 (IGF-1) level in urethane-induced lung cancer in BALB/c mice. (B) Effect of sildenafil on serum level of cytokeratin 19 fragments (CYFRA 21-1) level in urethane-induced lung cancer in BALB/c mice. Data were expressed as mean ± SEM; n = 15 mice per group. One-way ANOVA followed by Tukey-Kramer multiple comparisons test was used to compare means of different groups. *Significantly different from normal control at $p < 0.05$. [a]Means significantly different from urethane at $p < 0.05$.

in animals pre-treated with urethane crucially attenuated ICAM-1 and MMP-2 levels in comparison to urethane-treated group.

Effect of sildenafil on lung NF-κB, Caspase-3 and Nrf2 contents in urethane-induced lung cancer model in BALB/c mice.

Table 2 Effect of sildenafil on lung cGMP content in urethane-induced lung cancer in BALB/c mice

Treatments	Parameter, cGMP (ng/ml),($\bar{X} \pm$ SEM)
Normal control	13.57 ± 0.24
Sildenafil	17.23 ± 0.63 [a] *
Urethane	4.29 ± 0.11 *
Urethane + carboplatin	11.13 ± 0.49 [a]
Urethane + sildenafil	12.1 ± 0.74 [a]

Data expressed as mean ± SEM; n = 15 mice per group. One-way ANOVA followed by Tukey-karmer multiple comparisons test was used to compare the means of different groups.

*Significantly different from normal control at $p < 0.05$.

[a]Means significantly different from urethane at $p < 0.05$.

The results of this study, as shown in Figure 5, revealed that urethane treatment markedly induced NF-κB and Nrf2 protein levels then they were significantly suppressed in treatment with either sildenafil or carboplatin after urethane induction. Sildenafil reduced the level of NF-κB when compared to carboplatin after the treatment with urethane.

On the other hand, when the normal control and sildenafil groups were compared with the urethane group, it was observed that a remarked reduction in caspase-3 level in the urethane-treated group. Additionally, caspase-3 levels were significantly elevated in mice treated with either sildenafil or carboplatin after urethane induction without any statistical significance between both groups.

Effect of sildenafil on lung histopathological changes in urethane-induced lung cancer model in BALB/c mice

Examination of lung sections from the normal control group showed normal architecture of the lung with normal bronchi and bronchioles and normal alveoli. The vasculatures were normal and there were no signs of any tumour cells. H&E ×100 (Figure 6A).

Lung sections from the group treated with sildenafil only showed normal blood vessels with normal epithelization of bronchi and bronchioles and there were no tumour nodules. H&E ×100 (Figure 6B).

Animals that were treated with urethane showed aggregations of tumour cells around the bronchioles with polymorphism and advanced mitotic features. The epithelium cells of the bronchus were replaced by neoplastic cells (arrow) and the lumen was fully occupied with tumour cells (arrowhead). H&E ×200 (Figure 6C).

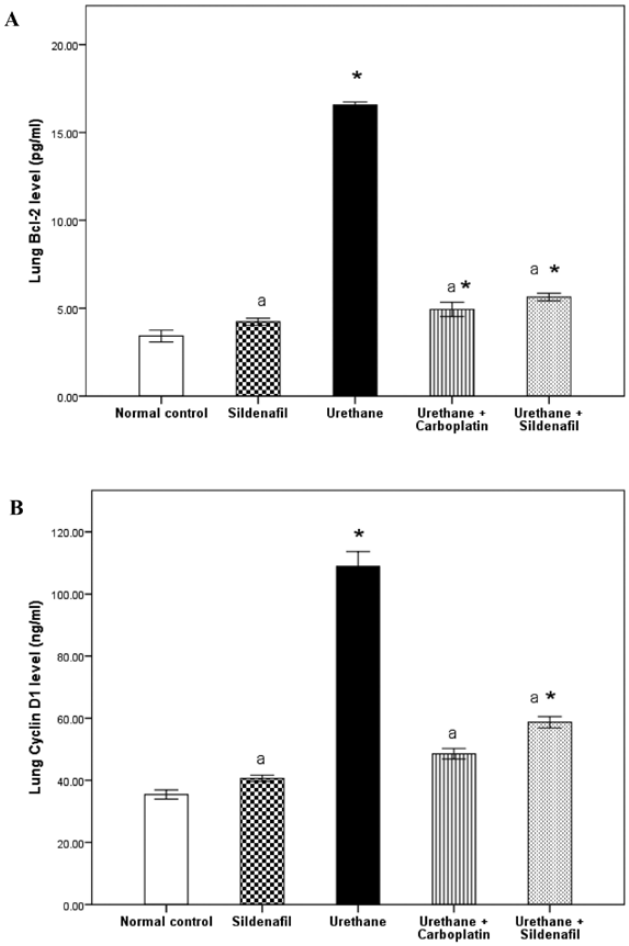

Figure 3 Effect of sildenafil on lung apoptotic and cell cycle biomarkers as Bcl-2 and cyclin D1. (A) Effect of sildenafil on lung Bcl-2 content in urethane-induced lung cancer in BALB/c mice. (B) Effect of sildenafil on lung cyclin D1 content in urethane-induced lung cancer in BALB/c mice. Data were expressed as mean ± SEM; n = 15 mice per group. One-way ANOVA followed by Tukey-Kramer multiple comparisons test was used to compare means of different groups. *Significantly different from normal control at $p < 0.05$. [a]Means significantly different from urethane at $p < 0.05$.

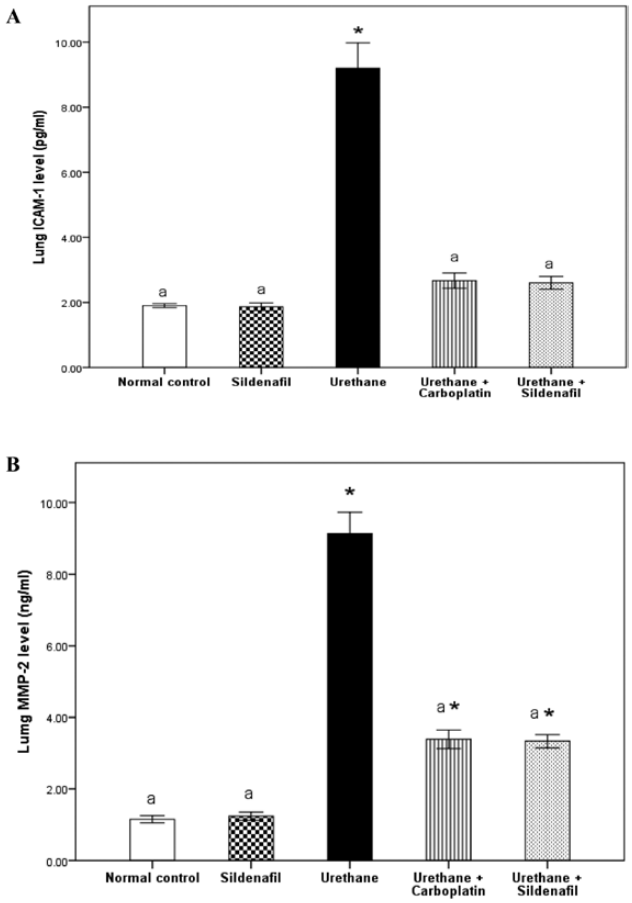

Figure 4 Effect of sildenafil on invasion biomarkers as ICAM-1 and MMP-2. (A) Effect of sildenafil on lung ICAM-1 content in urethane-induced lung cancer in BALB/c mice. (B) Effect of sildenafil on lung MMP-2 content in urethane-induced lung cancer in BALB/c mice. Data were expressed as mean ± SEM; n = 15 mice per group. One-way ANOVA followed by Tukey-Kramer multiple comparisons test was used to compare the means of different groups. *Significantly different from normal control at $p < 0.05$. [a]Means significantly different from urethane at $p < 0.05$.

Higher magnification of the previous slide showed additional clarified characters of malignancy of peribronchial (P) neoplastic cell aggregations (circle) with focal necrosis (triangle) and karyorrhexis (square). H&E ×400 (Figure 6D).

The lung sections from the group treated with carboplatin after urethane induction revealed few focal areas of adenocarcinoma (thin arrow) indicating incomplete recovery and the rest of the lung sections appeared normal in structure with normal alveoli and bronchioles (thick arrow). H&E ×100 (Figure 6E).

Mice treated with sildenafil after urethane-induced lung cancer manifested some peribronchial aggregations of inflammatory cells (star) with nearly normal appearance of lung section with normal (arrow) and dilated alveoli (arrowhead) showing compensatory emphysema. H&E ×100 (Figure 6F). The histopathological alteration in the lung was scored for all groups and presented in Table 3.

Discussion

Nowadays, there is a great interest to explore novel therapeutic agents able to overcome the severe adverse effects and acquired resistance that accounts in traditional chemotherapy and improve the patient's survival.

Earlier studies have been reported the potential role of PDE5 inhibitors, such as exisulind (sulindac sulfone), to be targeted in cancer treatment.[15] Additionally, exisulind was previously reported to inhibit lung tumour growth of chemically-induced carcinogenesis in rodents model.[44] Moreover, exisulind showed apoptotic activity of breast and colon cancer cells associated with PKG activation.[45,46] It worth noting that in vivo and in vitro studies with exisulind in NSCLC models showed remarkable induction of apoptosis contributing to its PDE5 inhibitory effect and subsequent β-catenin attenuation, mitogen-activated protein kinase 1 and c-Jun NH2-terminal activation which indicated by active caspase-3.[16] In addition, exisulind induces cell cycle arrest at G_1 phase in human lung cancer cells and its combination with docetaxel augmented the inhibition of cell growth and metastasis and improved animal survival.[47,48] However, phase I clinical study with oral exisulind alone (600 mg twice daily) in patients with advanced NSCLC showed severe toxicity indicated by elevation of liver functions; therefore, sildenafil may be more applicable in inducing almost the same mechanism with less adverse effects.[48] Sildenafil was shown to induce apoptosis in different types of cancer cells includes B-cell lymphoma,[49]

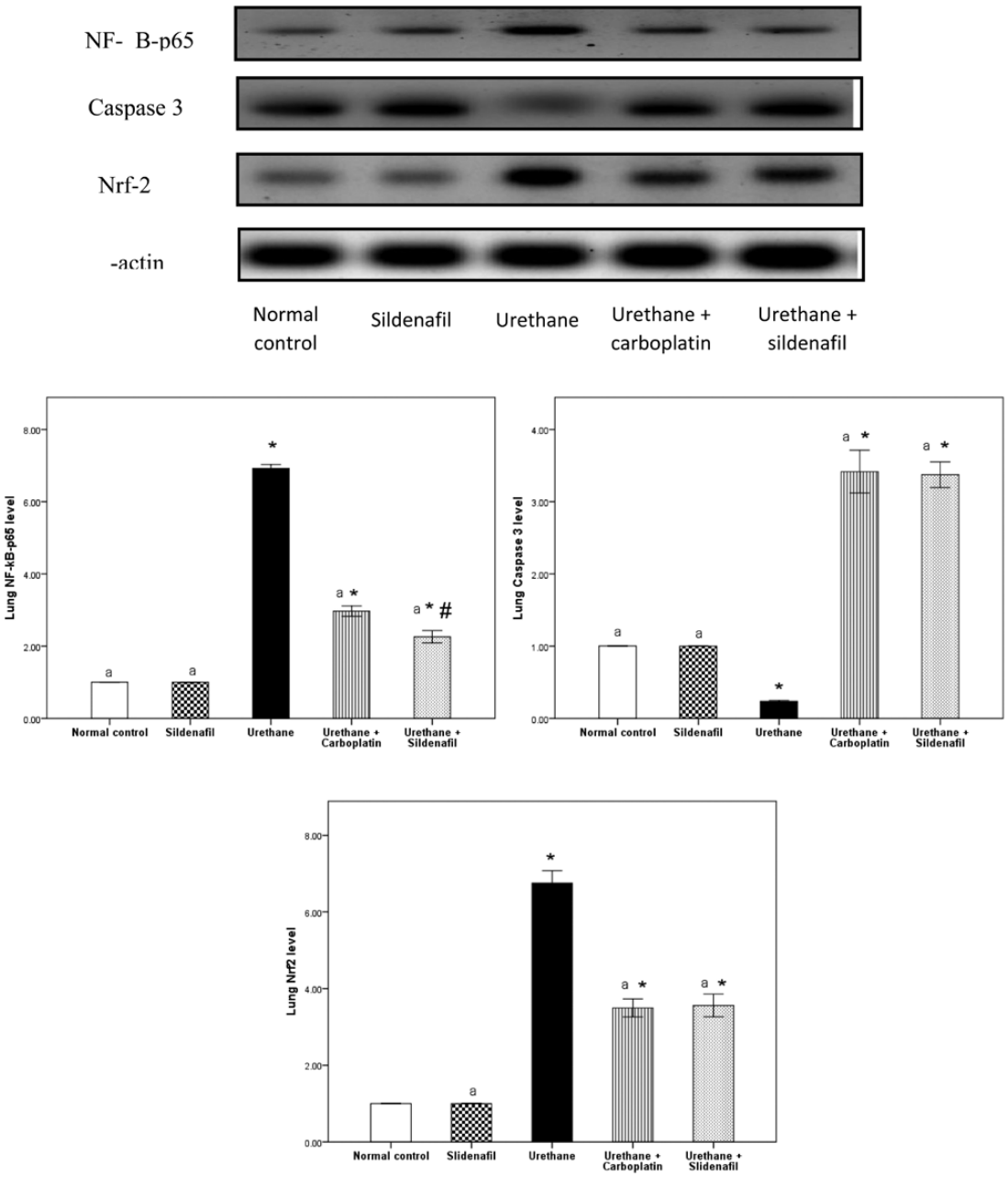

Figure 5 Effect of sildenafil on lung NF-κB, Caspase-3 and Nrf2 contents in urethane-induced lung cancer in BALB/c mice. Data were expressed as mean ± SEM; $n = 15$ mice per group. One-way ANOVA followed by Tukey-Kramer multiple comparisons test was used to compare the means of different groups. *Significantly different from normal control at $p < 0.05$. [a]Means significantly different from urethane at $p < 0.05$. #Significantly different from urethane + carboplatin at $p < 0.05$.

prostate,[50] colon[51] and colorectal cancers[18] as long as its vital role in enhancement of chemotherapeutic cytotoxicity in the breast,[52] brain,[53] bladder and pancreatic cancers.[54] Accordingly, these findings encouraged us to investigate the underlying molecular mechanism of sildenafil as a potential therapeutic regimen against urethane-induced lung adenocarcinoma in BALB/c mice.

The present results revealed that urethane (1.5 g kg^{-1}) induced lung adenocarcinoma evidenced by the outstanding elevation of serum level of IGF-1 and CYFRA 21-1. It has been previously reported that IGF-1 may be involved in the induction of lung cancer through the activation of phosphatidylinositol-3kinase (PI3K)/protein kinase B (AKT) pathway leading to inactivation of apoptotic proteins and enhancement of the anti-apoptotic protein Bcl-2.[55,56] Moreover, IGF-1 was found as one of the promoting factors in lung cancer progression through activation of mitogen-activated protein kinase (MAPK) that promote cell growth and proliferation via stimulation of ELK1.[57] In addition, CYFRA 21-1 is a specific tumour marker associated with poor prognosis and worsening overall survival of NSCLC.[58]

To express the mechanism of urethane-induced lung cancer, it may be introduced through persistent activation of Nrf2,[59,60] NF-kB[61] and anti-apoptotic protein Bcl-2 that is indicated by prevention of cellular apoptosis,[62] stimulation of ICAM-1 expression due to activation of NF-kB,[63] leads to activation of MMP-2[64] and accompanied with induction of cyclin D1 expression due to Bcl-2 in the distinct independent pathway.[65]

The results of this study showed no significant changes in animals treated with sildenafil except for a marked increase in cGMP level

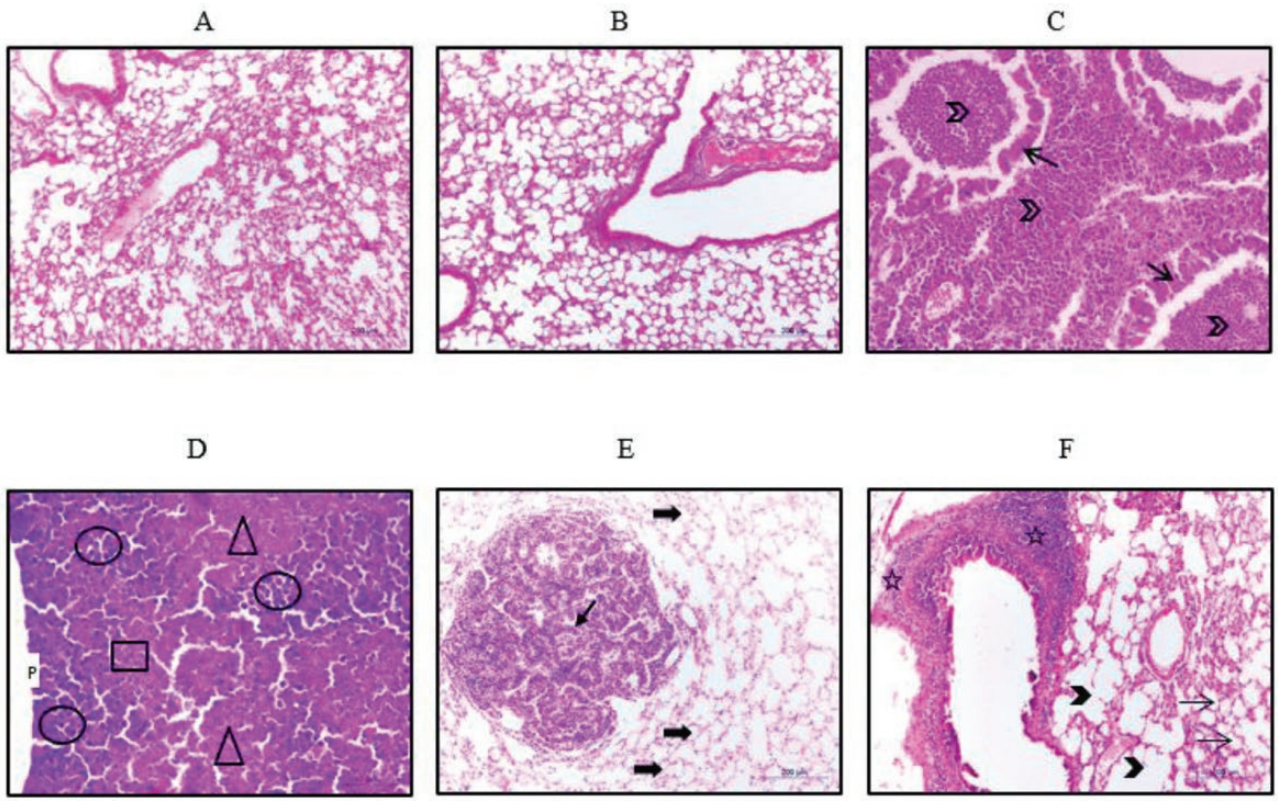

Figure 6 Effect of sildenafil on lung histopathological changes in urethane-induced lung cancer in BALB/c mice. Photomicrographs of lung sections stained with H&E stain. (A) Lung section from the control group with normal alveoli and vasculatures (H&E ×100); (B) Lung section of sildenafil group showing normal bronchi and bronchioles; (C and D) Lung sections of the urethane group showing intrabronchial and peribronchial neoplastic cells with mitotic features and aggregations of tumour cells (arrows) around the bronchioles (H&E ×200); (E) Mice treated with carboplatin after urethane showing incomplete recovery with normal vasculatures (H&E ×100); (F) Treatment with sildenafil after urethane showing some aggregations of inflammatory cells(arrows) around the bronchus and the rest of the lung section was nearly normal in structure.

Table 3 Histopathological effect of different treatments on the lung

Histopathologic alterations in lung	Normal control	Sildenafil	Urethane	Urethane + carboplatin	Urethane + sildenafil
Aggregations of tumour cells	–	–	+++	++	–
Advanced mitotic features	–	–	+++	+	+
Neoplastic cells	–	–	+++	++	–
Neoplastic cell aggregations	–	–	+++	++	+
Congestion of the vasculatures	–	–	+++	++	+

–, absent; +, mild; ++, moderate; +++, sever.

through the inhibition of PDE5.[66] Additionally, sildenafil treatment showed no significant changes in animals' body weight in comparison to the control group, while it showed a significant improvement in weight loss after urethane treatment. This is in full agreement with the safety data of sildenafil, which is a well-tolerated drug with mild adverse effects.[18,67] Sildenafil provides an erectogenic effect due to stimulation of cGMP with effective concentrations for 50% stimulation (EC50) 430–520 nmol/l in the rabbit corpus cavernosum.[68]

According to *Abou-Tarboush et al.*, the dose of sildenafil (50 mg/kg) is well tolerated and has been used in the treatment of erectile dysfunction or pulmonary hypertension dosage.[69] However, this dose is consistent with other investigators reporting that high dosages of PDE5 inhibitors are required for anticancer activity in the mouse tumour.[18] Specifically, this may be attributed to the overexpression of an alternative cGMP degrading PDE isozyme, PDE10, in lung tumours.[70] Regarding *Islam et al.*, up-regulated expression of PDE10 was suggested to be a compensatory mechanism overriding the highly specific inhibition of PDE5 by rapid degradation of arising cGMP levels consequently, high dosages to non-selectively inhibit other PDE isozymes that are co-expressed with PDE5 were required.[71] In support of this point of view, a dual inhibition of PDE5 and PDE10 isozymes was reported to have more robust anticancer activity.[72]

It worth noting that treatment with sildenafil after urethane induction exhibited a marked improvement in tumour incidence and multiplicity which is the context with previous studies reporting that sildenafil suppressed proliferation and inflammation of colon cancer cells.[71] Interestingly, sildenafil treatment after urethane-induced lung cancer showed a pronounced improvement of tumour markers (IGF-1 and CYFRA 21-1), apoptotic markers (Bcl-2, Cyclin D1) and a significant increase in caspase-3 and cGMP. These findings were indicated by induction of G1 cell cycle arrest and inhibition of tumour growth by the elevation of cGMP that activates PKG which down-regulates Wnt/β-catenin signalling

and Cyclin D1.[46] In addition, sildenafil induces Reactive Oxygen Species (ROS) generation that triggers caspases pathways leading to apoptosis.[54] The anticancer activity of sildenafil could be attributed to its properties in a reduction of NF-κB and Nrf2 activity and hence down-regulation of Bcl-2. These results are in context with the previous findings of the concerted modulation of NF-κB and Nfr2 to the normal value in lung tumorgenicity.[73,74] Contrarily, this result is in disagreement with the earlier study that showed sildenafil activates the anti-oxidative genes (Nrf2, HO-1 and NQO-1) with attenuation of pro-inflammatory cytokines (TNF-a, IL-1β and ICAM-1).[75]

NF-κB is a transcriptional factor in the kappa light chain of B cells. It plays an essential role in the regulation of cell proliferation, inflammation and invasion.[76] Previous investigations demonstrated that NF-κB up-regulates the expression of the anti-apoptotic Bcl-2 gene enhancing the cell survival that opposes the cytotoxic activity of chemotherapy[77] and up-regulates cyclin D1 in association with the transition of the cell from G1 to S phase.[78] In addition, NF-κB up-regulates MMP-2, which is responsible for extracellular matrix degradation and blood vessels invasion[79] and ICAM-1 has a role in cell invasion, metastasis and angiogenesis.[80]

The Nrf2-Keap1 pathway is one of the defence mechanisms against oxidative stress and xenobiotics. It governs the expression of antioxidants and detoxification enzymes that play a cytoprotective role against the chemical carcinogenic.[81,82] Recent studies revealed that Nrf2 is up-regulated in different malignant cells due to its pro-oncogenic role.[83] Interestingly, it was reported that Nrf2 directly up-regulates the expression of Bcl-2 and Bcl-xL associated with significant protection of cancer cells from apoptosis.[74] Therefore, the deactivation of Nrf2 is a potential target for cancer.[84]

The molecular mechanism of sildenafil may unveil through its multiple roles in the regulation of angiogenesis and metastasis that is indicated by a significant reduction of MMP-2 and ICAM-1. These results were in compliance with previous studies that indicated the role of MMPs and ICAM-1 in cancer metastasis and angiogenesis[85] and confirmed the anti-inflammatory effect of sildenafil through reducing intracellular adhesion molecules such as ICAM-1[86] and MMP-2.[87] The cytotoxicity effects of sildenafil may be due to a series of activation of several pathways including sequential activation of p38 and Extracellular signal-regulated kinases (ERK) pathways,[88] a noncanonical NF-kappaB activation,[89] stimulation of MAP kinase[90] and c-Jun N-terminal kinase (JNK)[91] by cGMP/PKG. Therefore, sildenafil may play an essential role in the induction of inducible nitric oxide synthase (iNOS) that showed several changes in cancer cells kinetics associated with anticancer activities[92,93] and enhance the chemosensitivity of tumour cells.[94] However, the antitumour activity of sildenafil may be attributed to DNA damage-induced by NO production, NO activates ataxia telangiectasia mutated (ATM) with inhibition of histone H2AX phosphorylation (γH2AX formation) leading to the promotion of pro-apoptotic signalling and irreversible DNA damage.[95] However, the role of NO in carcinogenicity remains controversial. At basal NO concentration, NO-dependent cGMP/PKG pathway tends to protect the cells from death while at higher concentrations, NO combines with superoxide anion forming toxic peroxynitrite favouring cytotoxic effect.[96] Some studies illustrated the anti-apoptotic role of NO/cGMP/PKG pathway in neural cells that may contribute to a low level of NO production induced through neuronal NO synthase isoform (nNOS) that previously well documented to produce cytoprotective small amounts of NO and subsequent low levels of cGMP.[97] The vital cytoprotective role of basal soluble guanylyl cyclase (sGC)/ cGMP can be approved by induction of apoptosis upon using selective inhibition of basal sGC and upregulation of p53.[98,99] These data were confirmed by enhancing the pro-apoptotic effect of high concentrations of NO donors through selective blocking of cGMP.[100] In contrast, a high concentration of NO was shown to be produced by inducible NOS (iNOS) isoform that may contribute to apoptotic effects shown with sildenafil.[92,101] Collectively, although NO (especially low and basal level) may show enhancement of tumour growth and angiogenesis in some types of cancer,[102] it also shows marked tumoricidal effects depending on NO concentration, final intracellular concentration, the type of cell exposed, duration of exposure and the redox state in the cancer cell.[103,104]

In addition, sildenafil showed a significant increase in protein expression of activated caspase-3 that may be due to its ability to damage the down regulatory mechanism of Bcl-2 that associated with caspase-3 activation.[105,106] The result of this work is in agreement with the earlier study that showed chemosensitizing activity due to doxorubicin in the prostate cancer cell and profound cardioprotective benefits.[50]

Carboplatin is the first-line and standard therapy in the treatment of non-small cell lung cancer, but the most toxic agent among other platinum compounds[107] because of the alteration of various cellular components. Carboplatin induces DNA adducts formation at N7 positions of guanine and adenine in intact DNA.[108] The results of this study showed that carboplatin treatment associated with a significant reduction of serum level of IGF-1 and CYFRA 21-1 that may be attributed to antitumour activity of carboplatin in lung cancer and positive impact against NSCLC.[109,110] However, the results of this study showed that carboplatin showed a pronounced improvement of all biochemical, molecular and histological examinations. These results are in agreement with earlier findings that revealed carboplatin induced SuperOxide Dismutase (SOD), Glutathione (GSH) activities and apoptotic gene expression in benzo[a]pyrene-induced lung cancer.[111] Surprisingly, carboplatin restores cGMP level which may contribute to excessive formation of ROS, especially hydrogen peroxide that is known to inhibit aggregation of different agents as a result of stimulation of guanylate cyclase and cGMP production.[112,113]

Histopathological examinations of animals treated with sildenafil and urethane confirm the biochemical and cellular examinations and showed an advanced improvement in the lung's histopathological patterns.

Conclusion

Collectively, results of this study provide novel evidence for drawing the molecular mechanism as anticancer that may attribute to sildenafil through activation of the cGMP/PKG pathway and down-regulation of cyclin D1 and elicits caspase-dependent apoptosis along with the reduction of the NF-κB pathway and hence their downstream of the anti-apoptotic gene Bcl-2.

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Author contributions

A.M.A. participated in writing a proposal and manuscript revision. M.M.K. participated in writing proposal and supervision during experiment conduction. M.N.M. conducting experiment and writing manuscript. M.A.H. wrote a

proposal, supervising during experiment conduction, performed data analysis, and manuscript revision and submission.

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Ethical approval

All experiments were accomplished according to approval released by Institutional Animal Care and Use Beni-Suef University (BSU-IACUC), Beni-Suef University (Permit Number: 018-43 at 4/8/2019) using the proper number of animals and unnecessary disturbance of animals was skipped meeting the three Rs' (Replace, Reduce, Refine) requirements. Animals were treated gently; squeezing, pressure, pain, malnutrition, abnormal cold or heat, injury, illness and tough manoeuvers were avoided.

Conflict of Interest

The authors declared that there is no conflict of interest.

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