A study to evaluate the effect of Ashwagandha (a phytoestrogen) on breast cancer, using MCF7 cell line.

Dr. Dabhade SA[1], Dr. Bhide HS[2], Dr.Tilak AV[3], Dr. Shaikh RM [4], Dr. Shah SN[5], Dr.Bapat SS[6*]

[1]Assistant Professor, Department of Pharmacology, Dr. D.Y Patil Medical College, Hospitaland Research Centre, Dr. D.Y Patil Vidyapeeth, Pimpri, Pune-411018

[2]Ex-Assistant Professor, Department of Pharmacology, Dr. D.Y Patil Medical College, Hospital andResearch Centre, Dr. D.Y Patil Vidyapeeth, Pimpri, Pune-411018

[3] Professor and Head of Department, Department of Pharmacology, Dr. D.Y Patil Medical College, Hospital andResearch Centre, Dr. D.Y Patil Vidyapeeth, Pimpri, Pune-411018

[4]2nd year postgraduate student, Department of Pharmacology, Dr. D.Y Patil Medical College, Hospital andResearch Centre, Dr. D.Y Patil Vidyapeeth, Pimpri, Pune-411018

[5]2nd year postgraduate student,, Department of Pharmacology, Dr. D.Y Patil Medical College, Hospital and Research Centre, Dr. D.Y Patil Vidyapeeth, Pimpri, Pune-411018

[6]2nd year postgraduate student, Department of Pharmacology, Dr. D.Y Patil Medical College, Hospital andResearch Centre, Dr. D.Y Patil Vidyapeeth, Pimpri, Pune-411018

*Corresponding author – Dr. Bapat SS
Email- shradsbapat@gmail.com
Mobile- + 91 9769835054

Abstract

Introduction

Breast cancer is the most common cancer in females all over the world. Traditional medicine suggests that plants containing phytoestrogens like Ashwagandha has some estrogenic effect. These have been claimed to have no side effects. Many of these phytoestrogenic plants have been reported to have anti-proliferative activity. Present study was planned to evaluate the role of Ashwagandha in different types of breast cancers.

Methodology

The effect of Ashwagandha on breast cancer cell lines was studied using MCF7 cell line. We evaluated the effect of Ashwagandha, tamoxifen and β-estradiol on ER positive cell line. compared the effects of Ashwagandha, tamoxifen and β-estradiol on ER (estrogen receptor) positive cell line. Also compared the effect of the combination of Ashwagandha + Tamoxifen with that of Tamoxifen alone on ER positive cell line.

Results

The survival rate reduced at all concentrations. Survival reduced even at lowest concentration and significantly reduced further with increasing concentrations of Ashwagandha. This indicated that the effect was dose dependent.

Conclusion

It was found that, the Ashwagandha has, significant dose dependent inhibitory action in MCF 7 cell line. Ashwagandha is devoid of any toxic effect and has the potential to evolve as a safer treatment option along with standard of care in breast malignancy.

Introduction

Breast cancer is the most common cancer in females all over the world. Its incidence was relatively higher in developed western countries of Europe and US. However recently, significant rise is seen in the incidence of breast cancer in many developing countries including Asian countries.[1-3]According to Ferlay et al., one third of the burden of breast cancer in the world has been found in India, US and China. There is big challenge for India about breast cancer because of rise in incidence rate and rise in mortality rate from 2008 – 2012.[4,5]According to therapeutic targets breast cancer is divided into three types. ER positive (most common with better prognosis) which grows by mainly ER signaling due to abundant expression of ER which is therapeutic target for SERMs.

HER positive (relatively less common with bad prognosis) which has abundant expression of HER-II receptors and grows mainly by HER-II signaling which is the therapeutic target of certain monoclonal antibodies like Transtuzumab and Imatinib. Third is triple negative (least common but having worst prognosis) which grows by different mechanisms working independent of ER and HER signaling and are commonly treated by doxorubicin.

Most important risk factor for breast cancer is related to estrogen status in the body. Thus, prolonged exposure of estrogen to breast throughout reproductive life is the main risk factor. Although it is uncommon, progesterone (P) can also increase the risk of breast cancer as its main site of proliferative action is acinar cells.

The definitive treatment of breast cancer is surgical excision with or without radiotherapy. However, chemotherapy is still required, before surgery in order to reduce the tumor mass, making it firm, more defined and thus easy to remove, after surgery and/or radiotherapy as an adjuvant in an attempt to improve prognosis and survival rate and as palliative treatment in those cases where surgery cannot be performed.

The current therapeutic agents in the management of breast cancer are divided into hormonal and non-hormonal agents. Non-hormonal chemotherapy is particularly effective in ER negative cases including doxorubicin, cyclophosphamide and others. The hormonal chemotherapy includes Selective Estrogen Receptor Modulators (SERMs) which have anti-estrogenic action on breast e.g. tamoxifen, toremifen and raloxifen and are particularly effective in ER positive breast cancer. Although these agents are relatively safer, still these have certain serious side effect e.g. tamoxifen and toremifen have potential to cause endometrial cancer as these agents have proliferative effect on the endometrium. All SERMs have risk of developing thrombo-embolic episodes, which can have disastrous consequences and hot flashes which are quite troublesome. Thus, there is need for the development of new agents with good efficacy and minimal adverse effects.

The phytoestrogens are plant derived compounds acting as agonist at some and antagonist at the other estrogenic receptors, thus acting as SERMs. There are many plants containing phytoestrogens and these agents are put to use in ayurvedic practice.

Ashwagandha i.e. *Withania somnifera* (Ashwagandha; Indian winter cherry, Indian ginseng) is abundantly available in India and is used as a medicinal plant. It belongs to the Solanaceae family of flowering plants. It has potential to treat menopausal syndrome[6] and is also reported to work in breast cancer.

Since Ashwagandha is used as agonist (in menopausal syndrome) and antagonist (in breast cancer), present study was planned to evaluate its role in ER positive breast cancer and also to find the reason for use of Ashwagandha as one of the therapeutic agents.

Materials & Methods

Cell lines:

- Selected cell line :
  - MCF7 in category of ER/PR positive cell line

- Procurement :
  - Cell lines were obtained from National Center of Cell Sciences (NCCS), which is an autonomous institution of the department of Biotechnology, Government of India. This is an authentic source for cell lines.

Cell culture media:

MCF7: Dulbecco's modified eagle medium

Supplements:

- 10% Fetal Bovine serum
- 2mM L-glutamine
- 50µg/ml streptomycin
- 50 IU/ml penicillin

These media and supplements were purchased from Sigma – Aldrich Chemicals Co., St. Louis Mi, USA.

Chemicals:

- MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]
- DMSO and Glycine buffer

These chemicals were also purchased from Sigma – Aldrich Chemicals Co., St. Louis Mi, USA.

Cell culture and evaluation of drugs:

96 well microplate:

It is a flat plate containing small wells in which cell suspension is added for incubation. The wells can be grouped according to test drugs and their serial dilutions. The test drug or extract of variable concentration was added into wells and kept for

certain incubation for 48 hours. Then its effect (proliferative or anti-proliferative) can be tested.

Extraction method:

The extraction methods were according to ayurvedic literature. .An aqueous extracts were made of plant material. The final ratio of plant material to water was 1:4. For roots of Ashwgandha the method was boiling. In Ayurveda, it is called as *manthan.*

The aqueous extract thus obtained was filtered through muslin cloth followed by centrifugation at 13000rpm at 4^0C. The filtrate was syringe filtered (0.25micron) and aliquots were stored at 80^0C after determining the concentrations.

Ashwagandha was dry powder. So, the concentration of the extract could be determined by knowing the amount of dry powder used to get the aqueous extract. For initial screening using in-vitro tests, 1000µg/ml concentration was used which was further sequentially diluted to get desired working concentrations.

Preparation of working solution:

- Initially the stock solution of standard and test drugs was prepared which was then sequentially diluted to get the desired concentrations.
- The concentration ranges for β-estradiol, tamoxifen, were 16.2, 32.4, 65, 125, 250, 500 and 1000 µM/ml.
- The concentration ranges for Ashwagandha are 16.2, 32.4, 65, 125, 250, 500 and 1000 µg/ml.
- The concentration range for Doxorubicin was 1.34, 2.68, 5.73, 10.75, 21.5, 42.5 and 81.5 µM/ml.

Evaluation of drugs:

- The MCF7 (ER/PR positive) was cultured in Dulbecco's modified eagle medium (DMEM) with added 10% Fetal Bovine serum, 2mM L-glutamine, 50µg/ml streptomycin, 50 IU/ml penicillin and were maintained at $37^{o}C$ in humidified air containing 5% $CO_{2.}$ This was done to achieve the adequate cell count.
- The cells grown were adherent to each other. Trypsin was added to the solution to break down the adhesion and make the cells free. With this, uniform cell suspension is obtained. This process is called as trypsinization.
- Then cells (in cell suspension) were seeded to microwells of the microplate in varying number and MTT assay done after 24 hours, to determine the adequate number of cells in the microwell. Well (containing defined number of cells) giving optical density range of 0.9 to 1.0 was selected for further studies.
- Selected number of cells were added to microwells and allowed to grow for 24 hours.
- Then aqueous solutions of standard drugs, test plants and their combinations with each other were added to the plate in **serial dilution in triplicate**.
- The cells were then incubated for 48 hours. This was followed by MTT assay.

MTT assay:

Principle:

The MTT is a complex biochemical reagent which is used for determination of cell survivability. It is a dimethyl thiazole derivative of tetrazolium bromide and is chemically known as [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]. Only living cells can take up MTT and are able to metabolize it into product called formazan (in crystal form) by mitochondrial reductase enzyme. In this reaction yellow

MTT is converted into purple formazan, so color of cell suspension changes from yellow to purple. The amount of formazan thus gives the cell survivability.

Steps:

- 10µl of 5µg/ml MTT was added to wells and wells were incubated for 5-6 hours.

- After incubation, formazan crystals were dissolved by adding 100µl of DMSO (dimethyl sulfoxide) and 25µl of glycine buffer.
- The amount of formazan was assessed by the intensity of purple color which was measured as optical density using spectrophotometer. This gave cell survival.
- Wells containing only DMSO (without addition of any drug) were used as vehicle control.

- The cell survival was converted into percentage survival rate (% survival) with the help of following formula while considering control as 100% survival rate.

$$\% \text{ Survival} = 100 \times \frac{\text{OD of test drug at given conentration}}{\text{OD of control}}$$

- The % survival was plotted against concentration for further analysis.

Statistical analysis

- Data was coded and entered in Excel sheet
- It was analyzed using Graph Pad prism version 6.
- Percent survival at each concentration of drug was obtained by comparing with control by unpaired T test.
- For comparison between the drugs, one way ANOVA followed by Dunnet's post hoc test was used.

Results

Results on MCF-7 cell line

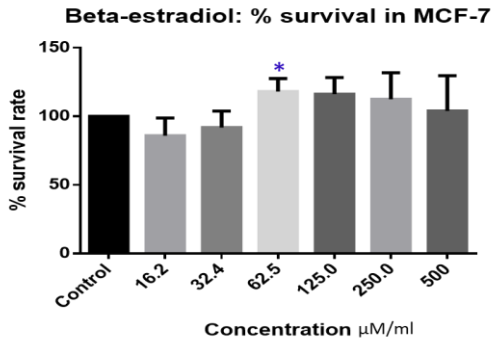

Figure 1: Effect of β-estradiol on survival rate

(Values were expressed as mean +/- SD,*p <0.05)

Unpaired‘t’ test was applied to compare mean of % survival rate at each concentration with that of control (as 100%)

The survival rate successively increased and it attained peak at concentration 62.5 µM/ml which was significant, after which survival rate showed gradual reduction. Overall, it showed proliferative effect.

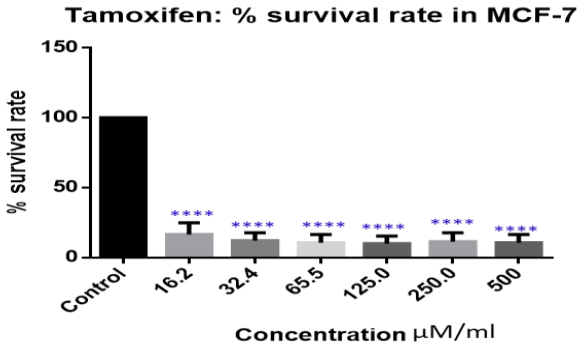

Figure 2: Effect of tamoxifen on survival rate

(Values were expressed as mean +/- SD , ****p < 0.0001)

Unpaired ‘t’ test was applied to compare mean of % survival rate at each concentration with that of control (as 100%)

Survival rate declined highly significantly at the lowest concentration and remained stationery with increasing concentrations. This indicated that the effect attains peak at very low concentration and is not dose dependent.

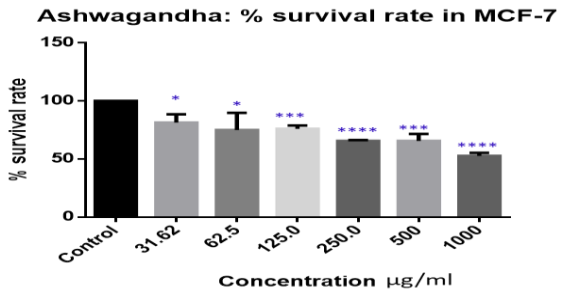

Figure 3: Effect of Ashwagandha on survival rate

(Values were expressed as mean +/- SD ,*p <0.05, ***p <0.001, ****p<0.0001)

Unpaired 't' test was applied to compare mean of % survival rate at each concentration with that of control (as 100%)

The survival rate reduced at all concentrations. As is seen in the above figure, survival significantly reduced even at lowest concentration and significantly reduced further with increasing concentrations of Ashwagandha. This indicated that the effect was dose dependent.

The comparison of effects of all drugs on survival rate :

Comparison was done between all the drugs tested on MCF-7 cell line. It was seen that β estradiol showed proliferative effect (more than 100%). Tamoxifen and Ashwagandha inhibited the survival. Tamoxifen had significantly more reduction in survival in comparison to Ashwagandha. Effect of the combination of Tamoxifen and Ashwagandha showed wide variation in the response and was not significantly different from Tamoxifen alone.

Discussion

Cancer is a leading cause of death worldwide and poses a challenge to the treatment. With overwhelming evidence of the role played by diet and lifestyle in cancer risk prevention, there is a growing interest into the search for chemo- preventative or chemo-therapeutic agents derived from natural products. This study was planned to ascertain the efficacy of Ashwagandha (phytoestrogen compound) in breast cancer MCF7 cell line.

Many times drug combinations have to be used for greater efficacy but the occurrence of adverse effects is the main limiting factor.

Breast cancers can be divided into ER positive, HERII positive and triple negative
We decided to work on ER positive breast cancer and selected cell line suitable for thesi type of cancers.

MCF-7- has estrogen receptor on its cells and is a common cell line to study the effects on ER positive breast cell malignancy.[7,8]So, MCF-7 cell line was used in the present study to evaluate the effects of the drugs on tumors having estrogen receptors. Estrogen receptor belongs to a nuclear receptor superfamily. It works through its receptors- ERα, and ERβ. Both these receptors are found in normal breast- ER β is more widespread in rodent & human breast in comparison to ER α [9, 10.] But as yet, its exact role is not clear. In breast cancers, ER α is upregulated in most of the patients whereas ER β levels are reduced. [10] Estrogen, after combining with estrogen receptor activates production of RNA- mRNA (messenger RNA), miRNA (micro RNA) and lncRNA (long non coding RNA). Out of these, mRNA and lncRNA promote synthesis of protein products leading to tumor formation. Remaining RNA exerts inhibitory action.

There are many receptors for estrogen action and there are many regulators of the action of estrogen (co-stimulators and co-repressors at the estrogen receptor level; agents acting at even post translational level). With widened insight into the action of estrogen, new treatment targets are getting identified for the development of new drugs. Though these details have evolved, still we treat ER positive breast cancers by

antagonizing estrogen hormone or its effects at the receptor level. Tamoxifen is Selective Estrogen Receptor Modulator (SERM) with antagonistic action on breast tissue and has become the standard approved agent for treating ER positive breast cancers.

Phytoestrogens are Selective Estrogen Receptor Modulators (SERMs) having agonistic-antagonistic action. To evaluate agonistic action of phytoestrogens, β estradiol was used.So, Tamoxifen, Doxorubicin and β estradiol were used as comparator agents to find the effects of test drugs.

Phytoestrogens are present in wide variety of natural substances and known to work like selective estrogen receptor modulators (SERMs). [11,12]Therefore, they have varying action profiles. In a study conducted on multiethnic population, Pamela L. Horn-Ross et al (2001) could demonstrate that consumption of phytoestrogens can reduce the chances of malignancy[13]. Use of herbal medicines is commonplace for Ayurvedic practitioners. Ashwagandha is a phytoestrogen, tried in the treatment of menopausal syndrome. Though some adverse effects are reported to phytoestrogens [14], as far as Ashwagandha is concerned, adverse effects are minor[15,16] which is the major advantage.

On MCF-7 cell lines, β estradiol produced proliferative effect. This is because of the agonistic action of β estradiol on estrogen receptors (ER-α) present on MCF-7 cell lines.

Tamoxifen, a synthetic agent, is SERM i.e. has agonist-antagonist action on estrogen receptor depending on the tissue concerned. It exerts antagonistic action on breast tissue. It has become a standard drug in the treatment of breast cancer and WHO has listed it as an essential drug in the treatment of breast cancer.[17,18] Importantly, this drug can also be used in the prophylaxis of breast cancer.

Tamoxifen significantly reduced cell survival in MCF-7 cell line.[19] In our study, Tamoxifen response was not dose dependent. Our result was different from the previous studies, in which dose dependence was seen in anti-proliferative activity of

Tamoxifen.[20] When looked into the details of the methodology, it was found that the authors had used lower doses of Tamoxifen than what we had used. In the present study, when lower dose of Tamoxifen was used (as in the combination of Tamoxifen and Ashwagandha- where the dose of Tamoxifen was reduced to half) the inhibitory effect on cell survival got reduced. The observed disparity could be because of the difference in the doses studied.

Ashwagandha (*Withania somnifera*), is used extensively for its medicinal properties in Ayurveda and Unani. It is also known as Indian ginseng, and as Indian Winter Cherry. This is an important ancient plant, the roots of which have been employed in the treatment. Withania species particularly grows in dry climate and is available all over the world. It has been traditionally used in Ayurveda as medicinal herb for menopausal syndrome. The roots of this plant have certain phytochemicals.

This plant is extensively studied. Twelve alkaloids, 35 withanolides and several sitoindosides have been isolated from the roots of the plant and researched for its medicinal properties. [28,29]. It is a non-toxic herb.[30] This herb is considered to be an adaptogen and is mainly used to normalize the physiological functions.[31]. It is used for a variety of clinical conditions like insomnia, osteoarthritis, improve complexion etc. It is used as neutraceutical in western world, but its medicinal properties are not recognized. Its anti-tumour activity was demonstrated as early as in 1967 [32], but clinical trials of Ashwagandha for this potential are in infancy, even in India, where it is used for its medicinal properties. Ashwagandha reduced survival in dose dependent manner, in MCF-7 cell lines . Similar results are reported by many authors [33, 34]. About 70% of breast cancers are positive for estrogen receptors (ER α type) and activation of these receptors play important role in the proliferation of these tumors. Negating the effects of estrogen, therefore, becomes the mainstay of the treatment for these cancers. Breakthrough came when Tamoxifen was discovered which reduced

the activation of these receptors and now it has become the mainstay of prevention and treatment of breast cancers, of course, ER positive ones. In the present study we compared the effect of Tamoxifen, Ashwagandha, and β estradiol on MCF-7 cell line. Tamoxifen was significantly more effective in reducing survival than other agents.

This probably was because Tamoxifen acts by inhibiting ER α receptors. Since growth in this tumor is mediated by ER α receptors, drug antagonizing the receptor has produced maximal efficacy. Ashwagandha was less effective in reducing survival in MCF7 cell line than Tamoxifen. The probable reasons could be, Tamoxifen effect is mediated through ER receptors to which it specifically binds whereas Ashwagandha might be working through some other pathway not involving estrogen receptors and Ashwagandha was used as an extract and not as a pure agent. Combining Ashwagandha and Tamoxifen – showed wide variation in the efficacy and produced effect similar to Tamoxifen alone indicating that there was no additional benefit of the combination.

This is very important finding which can have big clinical impact in developing effective yet safe drug combinations in the treatment of cancer.

Conclusion

Present study was carried out as a **"proof of concept"** study to evaluate the efficacy of phytoestrogen in Ashwagandha in MCF-7 (to consider action on ER positive breast cancer) breast cell line. It was found that, the Ashwagandha has, significant dose dependent inhibitory action in MCF 7 cell line. Ashwagandha is devoid of any toxic effect and has the potential to evolve as a safer treatment option along with standard of care in breast malignancy.

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