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Effect of two photo activated insecticides Intransglutaminase – mediated cell death on *Spodoptera furgipedra* Sf9 cell line *in vitro*

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Abstract: The *Spodoptera furgipedra* Sf9 cell line was tested with both Hematoporphyrin IX and B-Phenylpyruvic acid at LC_{50} 0.15 mM/ml and 0.35 mM/ml concentrations respectively. After irradiation of the tested cells, the photodynamic effect was measured with MTT assay. The results indicated that the B-phenylpyruvic acid and Hematoporphyrin IX were found to be effective as photo insecticides. In this cytochemical study we had tested the *Spodoptera furgipedra* Sf9 cell line after treatment with the previous photo insecticide for cytochemical evidence for transglutamase activity. The cells were treated in slide 8 well plat with gasket(8×10^4cells/well) after exposure to UV (380-400W/M^2) M^2 artificial lights) then we added the 0.5mM fluorescein cadaverine for 45min at $37C^O$, then fixed at $-20C^O$ in methanol. The slides were gently washed in PBS to remove any unincorporated fluorescein cadaverine. Remove wells gasket the cells were viewed under the fluorescent microscope (Zeiss Axioskop) for transglutaminase activity after adding mounting medium. The cytochemical results showed the activity of TGase enzyme due to the photodynamic effect of both B-phenylpyruvic acid and Hematoporphyrin IX. So we can conclude that these photosynthtizer were more effective to be used as a photo insecticide against *S. furgipedra*. The effect was not only due to singlet oxygen but also due transglutaminase mediated cell death on the treated cells which lead to apoptosis.. However the effect of B-phenylpyruvic acid was accompanied with losing of the cell integrity. This promising results have demonstrated great potentials to translate the morphological apoitosis to biochemical effect which is more precise in demonstration for applications in histopathological techniques to death diagnosis.

Keywords:Cytochemical, transglutamase, Hematoporphyrin IX, B-Phenylpyruvic acid, *Spodoptera furgipedra.*

Introduction

Some authors have reported the usefulness of hematoporphyrin as a photoinsecticides, [1-6]. Also The use of hematoporphyrin in insect control has several advantageous features as compared with conventional insecticides. This photoinsecticide can be directly administered in aqueous solution and in association with attractive; its photophysical, photosensitizing properties have been determined in a variety of media and have been shown to be particularly efficient,[7]. The primary physical process of photosensitization, the excitation of an electron, does not result in changing the spin of the electron, which is transferred to higher orbital. Therefore,

the excited molecule generated is in the singlet state. Lifetimes of excited singlet states are usually very short-measured in Pico-or nanoseconds, and therefore there is a chance of chemical reactions with other molecules (substrates) as reported by Halliwell *et al.* [8]. The relatively high water solubility of hematoporphyrin, the ascertained lack of photomutagenic activity, [9] and its wide spread clinical use as a phototherapeutic agent against solid tumours and other diseases, [10-11], although some potential hazards and limitation need to be considered,[2],[1],[3]. Photoinsecticide is also very rapidly photobleached upon exposure to UV or visible light, (9). Also,[12] reported the good use of photosensitizing Effects of Hematoporphyrin Dihydrochloride against the Flesh Fly *Parasarcophaga argyrostoma* (Diptera: Sarcophagidae).

The role of "tissue" transglutaminase (tTG) in apoptosis has been disscued by many others. Autuori et al. [13] reported that this enzyme by catalizing the Ca(2+)-dependent cross-linking of intracellular proteins leads to the formation of the SDS-insoluble protein scaffold in cells undergoing programmed cell death. These intracellular structures confer resistance to mechanical and chemical attack to the polipeptides involved in the linkages. tTG is induced during apoptosis, in fact, tTG mRNA is transcripted as a consequence of apoptosis induction. Overexpression of tTG in many cell lines enhances their susceptibility to apoptosis, indicating a pivotal role for tTG in this process. In keeping with these findings transfection of the human tTG complementary DNA in antisense orientation leads in a pronounced decrease of both spontaneous as well as induced apoptosis. Interestingly, the identification of the tTG substrate proteins in cells undergoing apoptosis has evidenced that many of the tTG proteins are also substrates of caspases. Also Nicholas et al. [14] stated that tissue transglutaminase (tTG) is a Ca(2+)-dependent enzyme which cross-links proteins via epsilon (gamma-glutamyl) lysine bridges. There is increasing evidence that tTG is involved in wound repair and tissue stabilization, as well as in physiological mechanisms leading to cell death. To investigate the role of this enzyme in tissue wounding leading to loss of Ca(2+) homoeostasis, we initially used a model involving electroporation to reproduce cell wounding under controlled conditions. Two cell models were used whereby tTG expression is regulated either by antisense silencing in ECV 304 cells or by using transfected Swiss 3T3 cells in which tTG expression is under the control of the tet regulatory system. Using these cells, loss of Ca(2+) homoeostasis following electroporation led to a tTG-dependent formation of highly cross-linked proteinaceous shells from intracellular proteins. Formation of these structures is dependent on elevated intracellular Ca(2+), but it is independent of intracellular proteases and is near maximal after only 20 min post-wounding. Using labelled primary amines as an indicator of tTG activity within these 'wounded cells', we demonstrate that tTG modifies a wide range of proteins that are present in both the perinuclear and intranuclear spaces. The demonstration of entrapped DNA within these shell structures, which showed limited fragmentation, provides evidence that the high degree of transglutaminase cross-linking results in the prevention of DNA release, which may serve to dampen any subsequent inflammatory response. Comparable observations were shown when monolayers of cells were mechanically wounded by scratching. In this second model of cell wounding, redistribution of tTG activity to the extracellular matrix was also demonstrated, an effect which may serve to stabilize tissues post-trauma, and thus contribute to the maintenance of tissue integrity.

It is very important to understand the proteases in insect. Transglutaminases catalyse the formation of covalent linkages between protein chains and can be used to restore the properties of the irreversible damaged proteins. However,although there are different ways in which cells may die, it is now thought that in a developmental context cells are induced to positively commit suicide whilst in a homeostatic context the absence of certain survival factors may provide the impetus for suicide. There appears to be some variation in the morphology and indeed the biochemistry of these suicide pathways; some treading the path of "apoptosis", others following a more generalized pathway to deletion, but both usually being genetically and synthetically motivated. There is some evidence that certain symptoms of "apoptosis" such as endonuclease activation can be spuriously induced without engaging a genetic cascade, however, presumably true apoptosis and programmed cell death must be genetically mediated. It is also becoming clear that mitosis and apoptosis are toggled or linked in some way and that the balance achieved depends on signals received from appropriate growth or survival factors.

Different types of cell death are often defined by morphological criteria, without a clear reference to precise biochemical mechanisms. The definition of cell death and of its different morphologies, while formulating several caveats against the misuse of words and concepts that slow down progress in the area of cell death research.

In this work we throw some light on the effectiveness of the both B-Phenylpyruvic acid and Hematoporphyrin IX as photosinsitizers to be used as a photo -activated insecticide. The survivability of the *S furgipedra* SF9 cell line due to B-Phenylpyruvic acid and Hematoporphyrin IX was determined by MTT (Tetrazolium day) assay. MTT was a colorimetric assay for anchorage depended cells, and was based on the permeability that MTT was taken up by viable cells and was reduced by the enzyme mitochondria dehydrogenase to yield purple formazan product, which was impermeable to cell membranes, resulting in its accumulation within viable /healthy cells. Solubilization of the cells by using Dimethylsulphoxide results in the release of the compound allowing its detection using a spectrophotometer. After tratment with B-Phenylpyruvic acid and Hematoporphyrin IX of the tested cells we investigated their lethal effect on transglutaminase activity cytochemicaly. For this reason we will get a full understanding of cell apoptosis on biochemical levells.

Experimental

1- Chemicals used

All chemicals of analytical grade were obtained from Sigma, Aldrich and Fluka (England). B-Phenylpyruvic acid was used. Stock solutions of B-Phenylpyruvic acid were prepared by dissolving a known amount of it in the minimal amount of ethanol. All different concentrations were made using PBS (Phosphate Buffered Saline) for dilution. Stock solutions of Hematoporphyrin were prepared by dissolving a known amount of Hematoporphyrin in the minimal amount of 0.1M Na OH and then neutralizing by drops of 36% HCL. The Hematoporphyrin concentration in the final solution was determined by absorption spectrophotometry, using $\varepsilon = 423000\ M^{-1}\ cm^{-1}$ at 401.5nm. The solutions of Hematoporphyrin were stable for 4 weeks when kept in the dark at 4^oC. Stock solutions were prepared by dissolving a known amount of each of them in distilled water. Preparation of the different concentrations were made using PBS (Phosphate Buffered Saline). All the above solutions were filter sterilized before use under hood.

2- Experimental models

S. furgipedra SF9 cell line was obtained from Invitrogen. This line was maintained for growth and subculture. This cell line originated from the IPLBSF-21cell line, derived from the pupal ovarian tissue of the fall army worm *S. furgipedra* [15,16]. These cells received as the following:

10^7 cells in 1ml 60%Grace`s medium, 30% FBS (fetal bovine serum), and 10% DMSO (Dimethylsulfoxide).

S. furgipedra SF9 cell line was maintained as an attached cell line at 27^0C in Grace's medium [55ml of FBS (fetal bovine serum) and 500 µl of a 10 mg/ml stock of gentamycin were added to 500 ml bottles of Grace's medium], this medium is stable for 3 months at $+4^0C$].

To determine the survivability of the *S. furgipedra* SF9 cells in presence or absence (control) of the tested photosinsitizers under UV or without UV (control), the following procedures were adopted according to Salama [17] as the following:

1. The cells to be trypsinised were washed 2x with 5ml of PBS (Phosphate Buffered Saline) and 4ml of trypsin (1x) were added per T-75 flask. The cells were then returned to 27^oC and 5% CO_2 for five minutes, in order to allow cell detachment.
2. After this time, 8ml of DMEM (Grace's medium [55ml of FBS (fetal bovine serum) and 500 µl of a 10 mg/ml stock of gentamycin were added to 500 ml bottles of Grace's medium]) was added to neutralise the action of the trypsin, and the entire volume of fluid was removed and transferred to a sterile centrifuge tube.
3. The cells were pelleted by spinning at 1200 rpm for 5 minutes. The supernatant was removed and the cells were resuspended in fresh media.100µl of this cell suspension was removed for cell counting using trypan blue staining. 200 cells were counted by using Hemacytometer and the number of cells per ml was calculated. The dilution ratio per well was calculated, using the following ratios:

-24-well plate, $5x10^4$ cells/well
-48-well plate, 2. $5x10^4$ cells/well

4. The cells were thus seeded and media to a final volume of 1ml for 24-well plate, 0.5ml for 48-well plate was added. The plates were then transferred to 27^{o}C and allowed to attach and grow overnight. Then the media were removed. 100µl of PBS were put per each well for washing with care. The different concentrations of the tested photosinsitizer were made from the prepared stock using PBS for dilution, 1ml was added to each 24- well plate, while 0.5 ml were added to each 48-well. Before irradiation, cells were incubated with the tested photosinsitizers for 1 hour at 27^{o}C

5. The UV lamp was turned on 10 minutes prior to irradiation, in order to allow it to equilibrate. Irradiation was carried out with the cells on ice (using an ice pack wrapped in tissue), and the cells were irradiated for 2o minutes. Silver foil was placed over the control wells.

6. At the end of irradiation the plastic cover was replaced and the cells were put under hood .The tested photosinsitizers and PBS were removed then washed with PBS. Immediate analysis was made immediately using either trypan blue cell counts or MTT assay.

Note: The percentage of cells mortality was plotted against the tested concentrations and the LC_{50} value were determined. Mortality percentage was always corrected by Abbott's formula [18] if the mortality in control exceeds 5%.

3- MTT Assay

The (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) Thiazolyl blue was used. According to Mosmann [19] and Yu Da, Yu Lin-Lin [20]. The S. furgipedra *SF9 cells (250.000/0.5ml) after treatment were subjected to the following:*

1- A 5mg /ml stock solution of MTT was freshly prepared in PBS, and filter-sterilized.
2- 50µl of the stock solution was added to 0.5 ml of media (48-well plate) and the cells were incubated for 1.5 hour at 27^{o}C.MTT was to be added to samples at a ratio of 10% of the volume of the sample.
3- After the incubation, the media was removed and the cells were washed with 100µl of PBS then can be viewed under the inverted microscope.1ml of DMSO was then added to the wells and the samples were agitated gently, and the absorbance of each well was read at 570nm immediately.

4- Cytochemical procedure

The *S. furgipedra* SF9 cell line was tested with the LC^{50} of the photosethtizer Hematoporphyrin IX and B-Phenylpyruvic acid on 8 well plate (8×10^{4}cells/well) with gasket. After exposure to UV (380-400W/M^2) M^2 artificial lights) then we added the 0.5mM fluorescein cadaverine for 45min at 37C^{O}, then fixed at -20C^{O} in methanol. The slides were gently washed in PBS to remove any unincorporated fluorescein cadaverine. Remove wells gasket the cells were viewed under the fluorescent microscope (Zeiss Axioskop) for transglutaminase activity after adding mounting medium. The control with the same procedure but without exposure to UVA lamp.

5- Irradiation

The artificial light source used was a Philips UVA lamp (HP3148/A, half body, 8TL09 lamps of 40W each, from Philips Electronics, Croydon) with an average output of $6.8x10^{5}$W cm^{-2} UVA and $6.1x10^{3}$ Wcm^{-2}UVB. The light intensity of the lamp was measured using a Glen Spectral Radiometer, model 1680B. The irradiation dose used was 7783 Jm^{-2}.

6- The statistical analysis

The statistical analysis was performed using t-test and analysis of variance (SPSS viersion 20).

Results

Table (1): Survivability of the *S. furgipedra* SF9 cells (250.000/0.5ml) due to the effect of the photosensitizer B-Phenylpyruvic acid or Hematoporphyrin IX with different concentrations after UV exposure (380- 400 W/M^2 artificial lights).

Concentrations (mM/ml)	Control without irradiation	Photosensitizer Hematoporphyrin IX	Photosensitizer B-Phenylpyruvic acid
Lc_{50}	0.0	0.15 mM/ml	0.35 mM/ml

Results of the present experiments are shown in Tables (1) and showeed in Figures (1, 2 and 3). The obtained data indicated that the cells survivability was highly affected by B-Phenylpyruvic acid and Hematoporphyrin IX as in Table (1) with Lc_{50}(0.35 mM/ml and 0.15 mM/ml) respectivly. The photodynamic effect was determined by MTT assay which found to be strong. The response is dose-dependent, i.e. the cells mortality increases with the increase of the concentration. The obtained data indicate also the following:

1. There is a significant increase in cells mortality due the effect of the B-Phenylpyruvic acid and the Hematoporphyrin as determined by MTT assay, ($p<0.05$).
2. The cell viability was greatly affected by Hematoporphyrin IX followed by B-Phenylpyruvic acid based on LC_{50} `s in Table (1) and shown in Figure (2&3).
3. The effect of L-b-phenyl lactic acid concentrations was not only affected the TGsae activity but also the cells integrity in significance level to induce cells apoptosis. This was confirmed also by trypan blue staining.

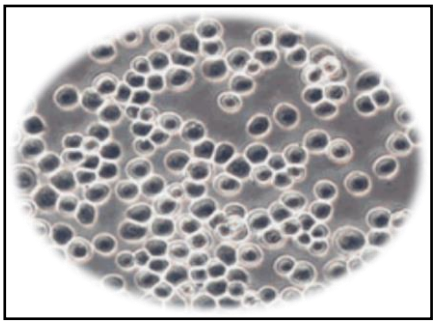

Figure 1: *Spodoptra furgipidra* Sf9 cells line as seen under phase contrast microscope healthy insect cells grown in suspension without any treatments (control). The culture was started in a shake flask at a seeding density of 3 × 10^5 viable cells/mL in Sf-900 II SFM medium and it was maintained in a 28°C, non-humidified, ambient air-regulated incubator. The images were obtained using 20 X objectives lens.

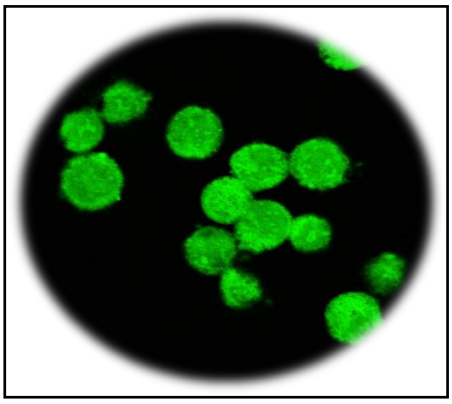

Figure 2: The cytochemical results showed that *Spodoptra furgipidra* Sf9 cells line as seen under the fluorescent microscope for transglutaminase activity after treated with Hematoporphyrin IX and irradiated with UV light and after adding mounting medium. The over expression of transglutamionase in the insect cells was clearly indicated which lead to the cell death. The images were obtained using 20 X objectives lens.

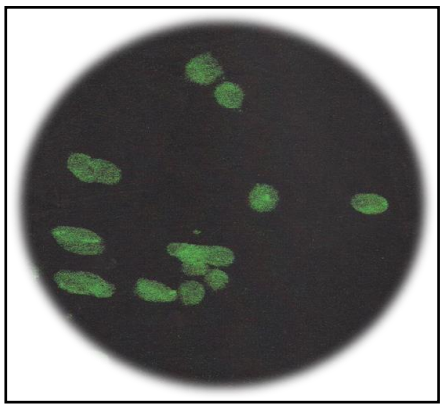

Figure 3: The cytochemical results showed that *Spodoptra furgipidra* Sf9 cells line as seen under the fluorescent microscope for transglutaminase activity after treated with B-phenylpyruvic acid and irradiated with UV light and after adding mounting medium. The over expression of transglutamionase in the insect cells was clearly indicated which lead to the cell death. The images indicated that the effect was accompaned with losing the cells integerty. The images were obtained using 20 X objectives lens.

Discussion

Apoptosis plays an important role in the considration of the good insecticide to be applied aganist insects. The use of *S. furgipedra* SF9 cell line to study the activity of TGase as this cells -free system provides a useful biochemical model to study the activation of apoptosis. The cytotoxicity testes indicated that the *S. furgipedra* SF9 cell line is quit susceptible to the photodynamic effect of both Hematoporphyrin IX and B-phenylpyruvic acid as measured by MTT assay. The reactive oxygen is greatly affected the cells as the concentration increases resulted in cells apoptosis (cell death). The results indicated that the use of *S. furgipedra* SF9 cell line is a good model for studying the susceptibility of this insect cell to the tested Photosensitizer. This finding agrees well with, [21] who reported that the cellular imbalance in the levels of antioxidants and reactive oxygen species (ROS) is directly associated with a number of pathological states and results in programmed cell death or apoptosis. Also with Salama [17] she reported in her *in vitro* studies that the Hematoporphyrin IX and B-phenylpyruvic acid were useful to be used as a photoinsecticides.

At the monents, its possible to conclude that the Hematoporphyrin IX and B-phenylpyruvic acid are a good photoinsecticides. The use of cell cultures for studying the effect of different insecticides instead of intact larvae provides several advantages, including reduced time, cost for experimentation. and absence of obscuring enzyme systems typically present in insects and a homogenous cell population lowering the strict measurement of insecticides effects. They demonstrated that the use of in *vitro* cultured *S. furgipedra* SF9 insect cells as a model to study oxidative stress induced programmed cell death. They added that apoptosis of in *vitro* cultured SF9 cells were induced by the exogenous treatment of H_2O_2 to cells growing in culture. The AD50 (concentration of H_2O_2 inducing about 50% apoptotic response) varied with the duration of treatment, batch-to-batch variation of H_2O_2 and the physiological state of cells. Also Kwa, *et al.* [22] concluded that the *S. furgipedra* SF9 insect cell line was most sensitive cell line towards the effect of the *Bacillus thuringiensis* delta-endotoxin Cry1C to cultured insect by means of toxicity assays. This finding was found to be in agreement with He Liming, *et al*. [23], in their test for comparing the photodynamic effect of 49 single laser photosensitizers on the SW1116 cell line of human colorectal cancer and K562 cell line of erythroleukemiwith MTT assay. They concluded the effectiveness of 16 single Photosensitizer from 49 one to be used in photodynamic killing of human cancer cell lines in *vitro*. Also these results confirm the findings of [9]. They concluded that the rapid insecticidal action of Hematoporphyrin could be correlated with its mode of photo inducing irreversible damage to biological systems. Our results agree will with, [4]. They pointed out that Porphyrins may represent a class of useful photoinsecticidal agents in particular, Hematoporphyrin appeared to be very active against at least two fly species, namely *Ceratitis capitata* and *Bactrocera oleae* which are known to induce severe damage in various agricultural areas worldwide.

The biochemistry of apoptosis is less well defined than its morphology, probably because this process charateristically involves scattered single cells within tissues, surrounded and outnumbered by viable neighbors. Nonetheless, six major events are known. Cell density rises abruptly [24-25]. Intracellular calcium concentration

undergoess a moderate but sustained rise (in necrosis, intracellular calcium rises rapidly several thousandfold)[26]. Total protein and RNA synthesis are shut down as stated by Wyllie and Morris[24]. Previously cryptic glycan groups become exposed on the cell membrane and act as recognition signals, permitting binding and engulfment by phagocytes as stated by Morris et al. [27] and Duvall et al. [28]. Cytoskeletal elements become less readly deformable, perhaps as a result of transglutaminase activity as stasted by Fesus et al. [29-30]. This was found to be in accordance with our results which indicated that the photoinsecticidal activity depends on the dose used as indicated by the LC_{50} `s of Hematoporphyrin IX and B-Phenylpyruvic acid (15 mM/ml & 35 mM/ml) respectively. Our cytochemical resluts confirmed the photodynamic phenomenon because its evidently increasing the transglutaminase acitivity in the treated cells. The present results indicated that Hematoporphyrin IX and B-Phenylpyruvic acid were very highly toxic to the tested *S. furgipedra* SF9 cell line as a photoinsecticide. The onsect of apoptosis is generally associated with a larg increase in enzyme synthesis and in its crosslinking activity as stated by (Fesus, et al., [30],[31-33]. The activation of TGase protein in dying cells results in the assembly of highly cross-linked intracellular protein. Di Sabatino et al.[34] stated that the Tissue transglutaminase (tTG) is a calcium dependent ubiquitous enzyme which catalyses posttranslational modification of proteins and is released from cells during inflammation.

In the future, there is much work to be done. Examples of some of the additional studies that are needed include research of the use of nanoparticles as indicator for cytochemical evidence of the tested materials including photoinsecticidal activity or any others insecticides which show the potential of the tested materials in inducing apoptosis, to make statistical cytochemical data in treated specimens.

The results confirmed the efficiency of both B-Phenylpyruvic acid and Hematoporphyrin IX as a photo-insecticide against *S furgipedra* with clear evidence in increasing the activity level of transglutaminase to induce insects cells death. Thus it may recommend the usage of the tested toxicants in control management strategy to reduce costs and pesticide impact in the environments.

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