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## The Establishment and Use of an *in vivo* Animal Model for Cervical Intra-Epithelial Neoplasia

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**Abstract:** Cervical cancer is the second most common cancer of female reproductive tracts. In developing countries, cervical carcinoma is the leading cause of cancer fatality in women. Despite attempts to lower the fatality rate, very few *in vivo* models are in place to investigate this cancer. We therefore are able to develop an *in vivo* animal model that is suitable to conduct such study. In our attempt to secure an *in vivo* animal model for cervical cancer, the carcinogenic property of diethylstilboestrol (DES) was exploited to establish a model for Cervical Intraepithelial Neoplasia or carcinoma (CIN). Female Balb/C mice were injected with several dosages of DES (i.p) during pregnancy at day 13-18. Female offspring were reared and sacrificed at age of 48-54 days and the cervix tissues taken for histological evaluation using H and E. The progression of the cancer and hence, disease state is monitored by measuring serum IL-6 using an ELISA kit. Proliferative cell nuclear antigen (PCNA) expressions were studied by implying immunohistochemical techniques. All parameters with regards to CIN were compared to a control group of treating the cancer using a used drug, cisplatin, used preferentially to treat cervical cancer in humans. The results of this study revealed that a significant difference in serum IL-6 concentration between DES-treated group and control groups ( $p < 0.05$ ). CIN histological related lesions was noticed to be prominently dominant in DES-treated animals whilst these lesions were absent in control groups. In addition to that PCNA index in DES-treated animal was found to be a significant different compared to control group. The above findings indicate that DES could be utilized and further exploited as cervical carcinogenesis initiator in animal models to screen and study new potential anti-cervical cancer compounds *in vivo*.

**Key words:** CIN *in vivo* models, carcinogenesis, cervical cancer, DES, cisplatin

## INTRODUCTION

Cervical cancer is a leading cause of mortality by cancer among female worldwide, with approximately 500,000 new cases and more than 200,000 deaths each year (Paul and Tiffany, 2005). Cervical cancer is considered to be refractory to endocrine treatment (Gabriella *et al.*, 2001). Prenatal estrogenic exposure alters the fetal genetic expression pattern of animal reproductive system, resulting in a characteristic molecular fingerprint. This is well-known example of adverse effects of chemicals with estrogenic activity on humans (Kaufman and Adam, 2002; Swan, 2000). Perinatal exposure of mice to DES generates a spectrum of reproductive tract lesions similar to those observed in humans (Takeshi *et al.*, 2004). Diethylstilbestrol (DES) is the first synthetic non-steroidal estrogen (Fig. 1). DES was synthesized in 1938 and was widely prescribed in the United States from the early 1940s

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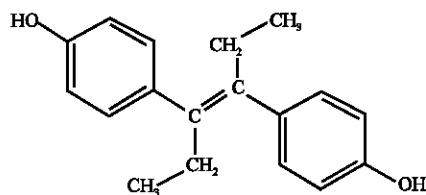


Fig. 1: Diethylstilboestrol

until 1971, primarily as a treatment to prevent miscarriages or premature deliveries. It has been banned by FDA in 1971 because of its link to a rare vaginal cancer (clear-cell adenocarcinoma) in diethylstilbestrol daughters (CDC, 2004). Finally it was listed in the First Annual Report on Carcinogens and by International Agency for Research on Cancer. The findings in humans are evidenced by studies in laboratory *in vivo* models (animals) showing that administration of diethylstilbestrol by various routes causes cancer in multiple species (mice, rats, hamsters, frogs, dogs and monkeys) and at multiple tissue sites (primarily estrogen-sensitive organs and tissues) (Mark *et al.*, 2005). CIN is a malignant tumor growing within the epithelium. It has been considered a pre-cancerous lesion. In CIN lesions, the number of layers of tumor cells increase and the order of the cell stratum is confused. Similar to the cells of invasive carcinoma, the cells of CIN typically show atypia: bizarre cell body, enlarged bulk of cells, enlarged nuclei with thick membrane and strongly stained pieces of chromatin, high frequency of mitotic figures and abnormal mitotic figures. CIN lesions are often demarcated from the adjacent normal epithelium. CIN I occupies one third of the epithelial stratum, CIN II two thirds and CIN III the whole epithelial layer. All CIN lesions have not yet broken through the basal membrane and never kill the patient because they do not metastasis (Burghardt, 1973; Quaas *et al.*, 2000). Research in cancer, related to development of new anticancer agents, needs biological models that ensure a valid, reliable, human related, environmentally friend, low cost aspects. These models can *in vivo* and *in vitro* research which must be done concurrently to introduce the new anticancer agents for further clinical evaluation.

## MATERIALS AND METHODS

### Animals

Forty inbred Balb/c mice, obtained from the Institute of Medical Research (IMR), Kuala Lumpur, Malaysia, were used in this study. Twenty female bal/b c mice were divided into 5 groups, each group consisting of 4 female mice. A single male mouse was placed into each group to stimulate ovulation for 48 h. After 48 h, the mice were separated into pairs, whereby 1 male mouse was cohabitated with 1 female mouse. The presence of the vaginal plug was examined the following day. The appearance of the vaginal plug is considered the start of pregnancy at Gestational Day (GD) = 0. When the pregnancies of female mice were confirmed, the pregnant female mice was separated from the male mice and house individually. Female Balb/C mice were injected with several dosages of DES (i.p) during pregnancy at day 13-18. Female offspring were reared and sacrificed at age of 48-54 days. Cisplatin and Normal Saline were as positive and negative control, respectively. This study was carried out according to the rules and regulations of Animal Care Unit Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia.

### Histology

For the histological examination, the cervical tissues from female mice offspring were fixed in 10% neutral buffered formalin immediately after removal from the animal, then dehydrated and embedded

in paraffin. Serial 4-5 mm cross sections were made through the cervix and stained with hematoxylin and eosin. To evaluate the associated histopathological CIN grades (I, II and III) were viewed under a light microscope.

### Immunohistochemistry

The immunohistochemical staining was conducted according to ARK kit (Animal Research Kit, Code K3954) in paraffin embedded cervical tissue sections. Briefly the endogenous peroxidase was blocked by Peroxidase Block (0.03% hydrogen peroxide containing sodium azide) for 5 min. The sections were then washed gently with wash buffer and then incubated with biotinylated primary antibodies for 15 min (PCNA and IL-6). It was followed by rinsing it gently with wash buffer and then placed in buffer bath. The slides were then placed into the humidified chamber and enough amount of Streptavidin-HRP (Streptavidin conjugated to horseradish peroxidase in PBS containing an antimicrobial agent) for 15 min and again rinsed gently with wash buffer and placed in buffer bath. DAB-Substrate-Chromagen (3, 3'-diaminobenzidine (DAB) + substrate buffered solution, pH 7.5, containing H<sub>2</sub>O<sub>2</sub> as preservative) was applied to the sections for 5 min. The sections were then washed with distilled water and the slides were immersed in coupling jar of hematoxylin for a length of 5 sec. Excess Hematoxylin was removed from the slides by running tap water and then dipped in weak ammonia (0.037 mol L<sup>-1</sup>) for 10 times and then rinsed in a bath of distilled water for a duration of 3 min. Specimens were mounted and coverslipped using DPX as non-aqueous permanent mounting medium.

### Serum Interleukin-6

Serum blood levels of IL-6, from female mice induced cervical cancer, was determined and quantitated using a commercial Anti-Mouse IL-6 ELISA Immunoassay kit after treatment with compounds, zerumbone, cisplatin and normal saline (positive control). The IL-6 serum levels in normal mice were used as negative control. Absorbance was compared to a standard graph of IL-6 immunoassay obtained (absorbance versus known IL-6 concentrations).

### Statistical Analysis

Data were expressed as the mean±standard error of the mean. One way ANOVA was used to analyze the difference between animals group using SPSS version 12. Confidence level was set at 95%.

## RESULTS

### Serum IL-6 Levels

Table 1 indicates that serum IL-6 levels of female mice induced cervical cancer treated with cisplatin were lower than mice induced cervical cancer treated with normal saline (p<0.01) and it is almost near to the serum level of normal mice (negative control). The Post Hoc comparison test (One-Way ANOVA). These results indicate that DES was capable to induce high level of IL-6 in CIN mice.

Table 1: Various concentrations of serum IL-6 with Standard Error Mean (±SEM), according to treatment groups in mice

Groups	IL-6 Mean±SEM (pg mL <sup>-1</sup> )
Normal	4.0±1.15
Cisplatin	6.0±3.79*
Normal saline	176.0±84.18*

\*Values are significant with p<0.01 analyzed by one-way ANOVA

### **Histological Findings**

Tissue sections of normal tissues showed normal epithelial arrangement with low nuclear: cytoplasmic ratio but with no presence of angiogenesis. In contrast to the normal saline treated female mice, cervical tissues of induced cervical cancer in female balb/c mice showed severe dysplastic changes (CIN III). In addition, the cervix tissues also exhibited arrangements of the epithelial cells to be disoriented (confined to whole layer), higher nuclear: cytoplasmic ratio (hyperchromatism) and cytoplasmic swelling and clarity with marked angiogenesis. Treatment of cervical cancer induced in female balb/c mice with  $10 \text{ mg kg}^{-1}$  of cisplatin showed mild dysplastic changes (CIN I), with mild cytoplasmic clarity, disoriented epithelial cell arrangements and mild angiogenesis to the cervix sectioning. This indicates that DES was able to produce histological lesions in the cervix that able to be reversed by a chemotherapeutic drug (Fig 2-4).



Fig. 2: Micrograph showing normal cervical epithelial cells with low nuclear: cytoplasmic ratio, without noticeable angiogenesis

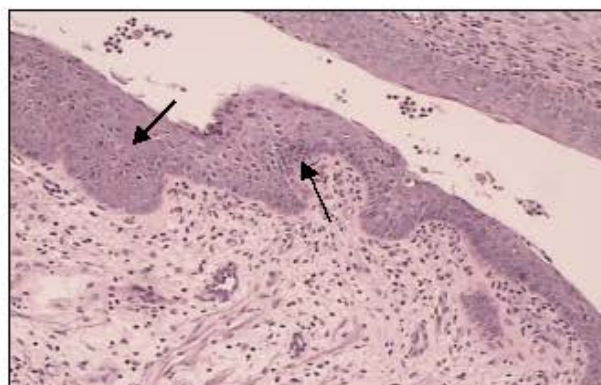


Fig. 3: Micrograph showing treatment of induced cervical cancer in female balb/c mice with normal saline. Severe dysplasia (CIN III), enlarged hyperchromatic nuclei with cytoplasmic swelling and clarity involving almost the whole layer of the epithelia (Black arrows) was noticeable. Micrograph was at magnification 10x20

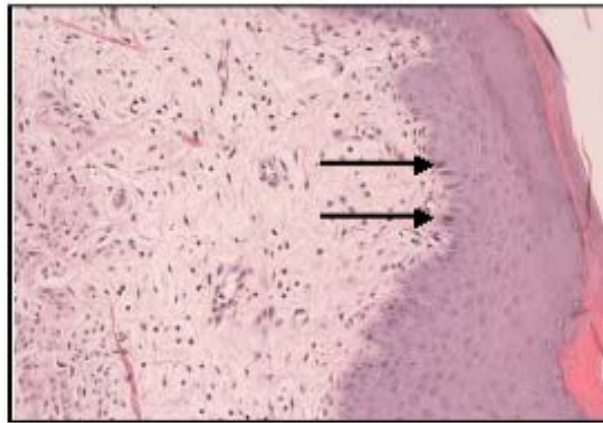


Fig. 4: Micrograph showing treatment of induced cervical cancer in female balb/c mice with  $10 \text{ ng kg}^{-1}$  cisplatin. Mild dysplasia (CIN 1), with mild nuclear atypia confined to one third of the epithelial layer (Black arrows) at magnification  $10 \times 20$

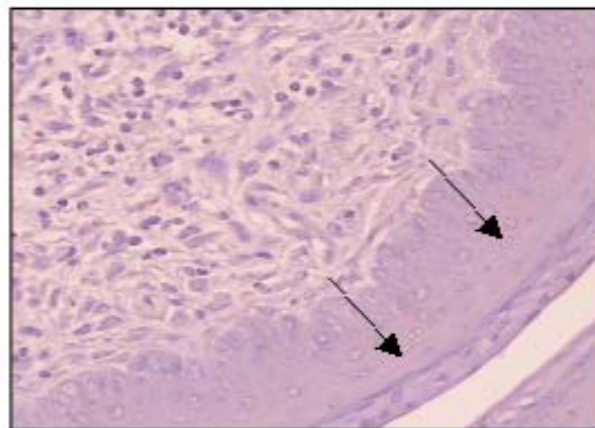


Fig. 5: Micrograph of cervical tissue of normal female mice containing very few brown pigments-black circle and square (IL-6 receptors). Instead, normal epithelial arrangement was noticeable. (Magnification  $10 \times 20$ )

#### **Detection of Surface Membrane Interleukin-6 Receptor (IL-6R)**

Immunohistochemistry technique was used to detect presence of cytoplasmic and membrane bound IL-6 Receptors of cells in female mice cervical cancer tissues after treatment with cisplatin. Immunohistochemistry micrographs showed that the IL-6R was expressed (brown pigments as viewed under phase contrast microscope) on membrane and cytoplasm of the cells in all treatment groups. However, a decreased to the intensity of staining (brown pigments) were noticeable in cervical cancer tissues of mice treated with cisplatin (Fig. 6) as compared to cervical cancer tissues of mice treated with normal saline (Fig. 7), the later relatively, had higher intensity of brown pigment. The cervical tissue of normal mice shows very low intensity of brown pigments, indicating that only few IL-6Rs were present (Fig. 5).

Table 2: PCNA labeling index cervical intraepithelial neoplasia experimental and control animals (Mean±SEM)

Treatments	PCNA labeling index
Normal control	5.0±2.20
CIS 10 mg kg <sup>-1</sup> b.wt.	6.0±3.30*
Normal saline+Cancer	60.0±2.30*

\*Statistically significant different at the probability level of 0.05. Post-hoc multi-comparison analysis was performed

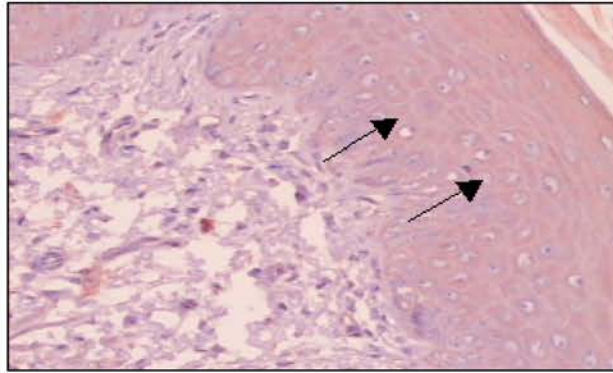


Fig. 6: Micrograph of cervical cancer tissue of female balb/c mice treated with cisplatin at 10 mg kg<sup>-1</sup> dosage. Fewer brown pigments (corresponding to surface IL-6Rs) were noticeable (black arrows) (Magnification 10x20)

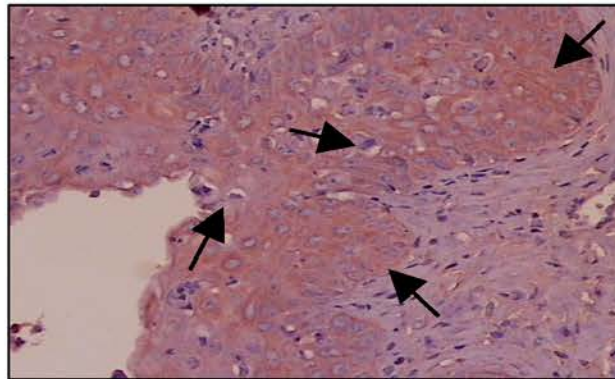


Fig. 7: Micrograph of cervical cancer tissue of female balb/c mice treated with normal saline. Higher intensity of brown pigments (black arrows) (corresponding to surface IL-6Rs), enlarged hyperchromatic nuclei (blue arrows) and cytoplasmic with swelling clarity almost covering the entire layer of epithelia were noticeable at magnification 10x20

#### Expression of PCNA and PCNA Index

The expression of PCNA in proliferating tumor cells can be visualized as a brown stain in nuclei but not in the cytoplasm because PCNA is a nuclear protein and is close correlative to DNA replication. Whilst PCNA-negative nuclei were counterstained by hematoxylin. PCNA positive nuclei were much higher in DES induced CIN tissues compared to cisplatin group and normal group (Fig. 8-10). PCNA index was statistically ( $p<0.05$ ) higher in DES induced group compared to normal and cisplatin groups (Table 2).

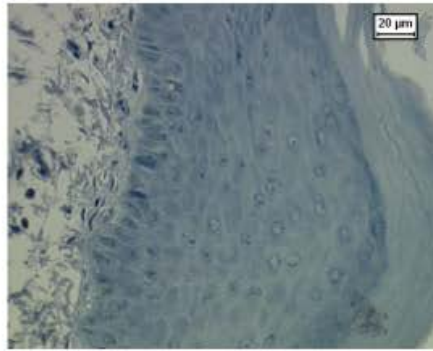


Fig. 8: Immunohistochemical staining of cervical tissue from normal female Balb/c mice using PCNA mouse monoclonal antibody and ARK immunohistochemical kit. PCNA immunoreactivity was not found in the nuclei (200X)

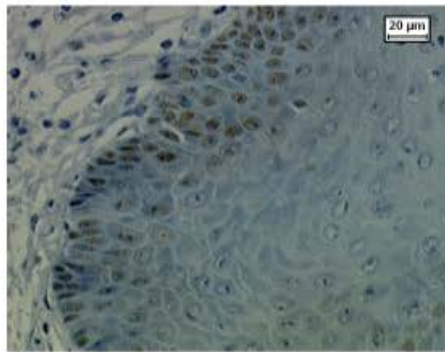


Fig. 9: Immunohistochemical staining of cervical cancer tissue, from female Balb/c mice treated with ARK immunohistochemical kit with the primary antibody (PCNA mouse monoclonal antibody). PCNA immunoreactivity was found in the nuclei (200X)

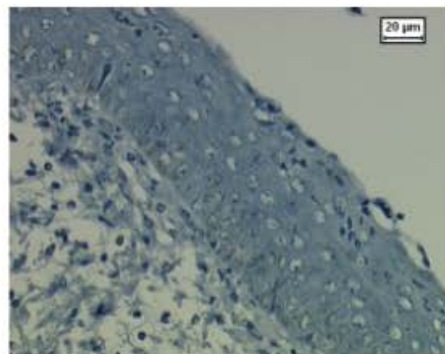


Fig. 10: Immunohistochemical staining of cervical cancer tissue, from female Balb/c mice treated with  $10 \text{ mg kg}^{-1}$  of cisplatin, using PCNA mouse monoclonal antibody and ARK immunohistochemical kit. PCNA immunoreactivity was found in the nuclei (200X)



## DISCUSSION

It has been shown that long-term use of hormonal contraceptives tends to increase the risk of cervical neoplasia (Jeffrey *et al.*, 1996; Magnusson *et al.*, 2000). With respect to that, DES was proven to cause cancerous lesions in daughters of mothers with previously using DES (Herbst *et al.*, 1971; Sarina and Beth, 2004). This research was carried out on the basis of DES carcinogenicity (Marselos and Tomatis, 1992; Ma *et al.*, 1998; Block *et al.*, 2000). Moreover, *in vivo* models are badly needed to study potential chemopreventive agents. In this study DES was intraperitoneally administered to pregnant female balb/c mice with hypothesis of that DES will generate noticeable and diagnosable lesions in cervical tissues of that respective female offspring. The obtained results of H and E micrograph showed that cervical tissues of DES administered female balb/c mice showed severe dysplastic changes (CIN III). In addition, the cervix tissues also exhibited arrangements of the epithelial cells to be disoriented (confined to whole layer), higher nuclear: cytoplasmic ratio (hyperchromatism) and cytoplasmic swelling and clarity with marked angiogenesis. Treatment of cervical cancer induced in female balb/c mice with 10 mg kg<sup>-1</sup> of cisplatin showed mild dysplastic changes (CIN I). These results indicate that DES was able to induce morphohistological lesions that can be prominently noticed with a prognostic property. These prognostic properties can be evidenced by the fact that cisplatin as anticancer agent was able to regress the progression of CIN lesions by using H and E which has been used experimentally and clinically to evaluate CIN lesions (David *et al.*, 2000; Anna, 2003; William *et al.*, 2004). To confirm the proliferative stimulation of DES on female offspring, immunohistochemical staining of PCNA was conducted. PCNA has shown a higher immunoreactivity in DES administered animals compared to normal and cisplatin treated animals. Whereby, PCNA could be used as proliferation index and be detected by immunohistochemistry (David *et al.*, 2000). H and E and PCNA results are indicating the tendency of DES to possess a higher proliferating potency in tumor cells. This fact can be utilized scientifically as a basis for *in vivo* model for CIN; moreover, it can be supported by the ability of cisplatin to regress these biomarkers. In addition to that PCNA scores were quantitatively and statistically compared between study groups whereby a significant difference was noticed between DES administered and DES+cisplatin animals. These results showed that DES was able to induce hyperproliferation of cervical epithelia. PCNA as a proliferation marker is being used for the diagnosis of cervical cancer (Tjalma *et al.*, 2001). Up-regulation of proliferating cell nuclear antigen (PCNA) is closely associated with progression of Cervical Intraepithelial Neoplasia (CIN) (Branca *et al.*, 2007). In addition to that PCNA was also used in certain studies that utilized diethylstilboestrol as an experimental carcinogen (Gail *et al.*, 2001). The quantification of PCNA immunohistochemical staining was performed as biological marker to evaluate the effect of new pharmacological agents in cervical cancer proliferation (Stepien *et al.*, 2006). Moreover it was also used to evaluate the anti-cancer properties of potential new drugs in various types of cancer (Morioka *et al.*, 2005).

Serum IL-6 levels of female mice induced cervical cancer treated with cisplatin were lower than mice induced cervical cancer treated with normal saline ( $p < 0.01$ ) and it is almost near to the serum level of normal mice (negative control). To correlate evidently the serum presence of IL-6 immunohistochemical detection of IL-receptor was carried out. Immunohistochemistry micrographs showed that the IL-6R was expressed (brown pigments as viewed under phase contrast microscope) on membrane and cytoplasm of the cells in all treatment groups. However, a decreased to the intensity of staining (brown pigments) were noticeable in cervical cancer tissues of mice treated with cisplatin as compared to cervical cancer tissues of mice treated with normal saline, the later relatively, had higher intensity of brown pigment. The cervical tissue of normal mice shows very low intensity of brown pigments, indicating that only few IL-6Rs were present. IL-6 plays an important role in the

development of cervical cancer by demonstrating that local over expression of IL-6 and its receptors occurs in cervical tissues that undergo active angiogenesis (Wei *et al.*, 2001). Moreover IL-6 is used as a prognostic indicator of cervical cancer (Srivani and Nagarajan, 2003).

### CONCLUSION

In conclusion, *in utero* exposure of DES in balb/c mice could be used as *in vivo* models to produce female offspring with cervical intraepithelial neoplasia. This model is identified by the reversibility of neoplastic lesions induced by DES because cervical carcinogenesis occurs in a stepwise fashion. The changeover of normal epithelium to preneoplastic lesions and invasive carcinoma occurs serially. The morphologically defined steps of dysplastic and malignant abnormalities are a reflection of cellular gene alterations during tumorigenesis. Future efforts in using molecular techniques are needed to verify the mode of action of DES and cisplatin. Finally this cervical intraepithelial neoplasia in model could be utilized in the screening of potential chemotherapeutic and chemopreventive agents.

### ACKNOWLEDGMENTS

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