The Egyptian Journal of Hospital Medicine (Apr. 2015) Vol. 59, Page 217- 226

Effect of Prenatal Exposure to Bisphenol A on the Endometrium of Albino Rats: a Histological and an Immuno-histochemical Study

Essam Eldin A. Salama[1, 2], Ali H. Ali [1, 4,] Tarek A. El Ghamrawy[3],Mohamed S. Farag[4], Lotfy S. Mohamed [5]

1-Department of Anatomy, Faculty of Medicine, Al Azhar University
[2-]Department of Anatomy, Faculty of Medicine, King Saud University, Saudi Arabia nm
3-Department of Anatomy, Faculty of Medicine, Cairo University
[4-]Department of Histology and Anatomy, Faculty of Medicine,
Salman bin Abdulaziz University, Al-Kharj, Saudi Arabia.
[5-]Department of Histology, Faculty of Medicine, Al Azhar University

ABSTRACT

Aim of the work: bisphenol A (BPA) is a synthetic monomer that is polymerized to manufacture polycarbonate plastic products and resins, including those used in food packaging and dental sealants. It is used in the manufacture of a great variety of products including: compact disks, food can linings, plastic windows, car parts, adhesives, protective coatings and powder paints. This work aimed to study the effect of prenatal exposure to BPA on the endometrium of female rats.

Materials and methods: thirty adult female albino rats were divided into three groups: rats in group 1 served as a control (G1) and received an equal amount of sesame oil to those of the treated groups; those in group 2 (G2) were administered by gavage 5.0 µg BPA/kg/day (low-dose group); the third group (G3) received 50 µg BPA/kg/day (high-dose group). The female offspring of each group were weaned at day 21 and maintained until 3 months old. The uteri were dissected for the histological and immuno-histochemical examination.

Results: low-dose group showed degeneration of the epithelial lining of the endometrium with focal patches of increased epithelial cell layers. The high dose group revealed cytoplasmic hydropic degeneration and pyknotic nuclei of the epithelial cells. Estrogen receptors showed a significant decrease of positive cells in low dose treated group and this decrease markedly accentuated in the high dose one. Positive nuclei for Ki-67 were markedly increased with increasing doses of BPA.

Conclusion: BPA showed obvious endometrial degenerative and proliferative histological changes. Therefore, the use of this substance in food packaging materials and in the manufacture of substances liable to come into contact with food and drink should be phased out.

INTRODUCTIION

The use of synthetic chemicals exerts a great influence on the daily life of human beings. Some of these synthetic chemicals can act as endocrine disrupting substances in various organisms. The reproductive cells are the most sensitive to toxic environmental materials. Bisphenol A (BPA) is a synthetic monomer that is polymerized to manufacture polycarbonate plastic products and resins, including those used in food packaging and dental sealants [1]. Many countries throughout the world have large production capacities of BPA, especially Germany, Netherlands, USA and Japan.

BPA induction of alterations in the male and female reproductive integrity has been shown in different species. Inhibition of the development of seminiferous tubules and spermatogenesis were observed after BPA treatment in the male chick [2,3].Toxic effects of BPA were observed in female reproductive system of the brown trout [4]and mussels [5,6].BPA induced morphological transformation, aneuploidy and DNA adducts in Sirian Hamster embryo cells [7]. It also suspected of causing birth defects of the reproductive tract (including un-descended testes) and other hormone related effects, such as earlier puberty in girls [8].

DOI: 10.12816/0012179

The mode of action of BPA at low levels appears to mimic that of the female hormone, estrogen. BPA therefore belongs to a group of chemicals termed "hormone disruptors" or "endocrine disruptors" that are able to disrupt the chemical messenger system in the body. There is growing international concern about manmade endocrine disrupting chemicals (EDCs), because they can de-rail the development of offspring exposed in the womb [9].

Experimental animal models have shown an estrogenic effect of BPA, and thus an endocrine disrupting action that may have long-term effects on the endocrine system, influencing tumour development later in life [10]. Prenatal exposure to BPA has been associated with hormonal, morphological, functional and behavioural anomalies related to reproduction [11]. It was demonstrated that rodent strains can vary dramatically in their response to estrogenic compounds. Furthermore, the issues of dose and binding affinities to the estrogen receptors (ERs) seem to be the heart of the controversy regarding xeno-estrogens [12].

The present work aimed to study the histological and immunohistochemical effect of prenatal exposure to BPA on the endometrium of adult female rats.

MATERIALS AND METHODS

Experimental animals:

The present study was carried out on 60 adult (20 males and 40 females) albino ratsweighing 180–250 g. Male and female rats were inbred in polysulfone cages,42 × 26 × 15 cm..They were maintained under a controlled environment and given rodent pellets and water *ad libitum.* The concentration of phytoestrogens in the diet was not evaluated because food intake was equivalent for the control and experimental rats and both groups were exposed to the same levels of phytoe-strogens [13]. Two females and one male were housed in a cage. During routine breeding checks, 30 female with noticeably sperm-positive uterus smears were removed andplaced in a separate cages.

Experimental design:

Pregnant rats were divided into three groups (10 per group). Group1 (control group) received an equal amount of sesame oil to those of the treated groups. Group 2 (low-dose group) was administered by gavage 5.0 µg BPA/kg BW dissolved in sesame oil. Group 3(high-dose group) received 50 µg BPA/kg BW dissolved in sesame oil [14].The treatment was performed daily from day 10 to day 20 post-conception. The female off-springs of each group were weaned at day 21 and maintained until 3 months old. Those female rats were followed before scarification to determine if they were undergoing a normal estrus cycle by vaginal smear using Shorr's stain solution. To define the phase of estrus cycle, every morning from 9–11 am vaginal smears were taken and processed[15].During the diestrus phase, the rats were euthanized by intraperitoneal injection of 75 mg/kg pentobarbital. The uteri were dissected for the histological and immune-histochemical examination.

Histological and immuno-histochemical technique:

Small pieces of the uteri were taken; each was divided into two parts. One part was fixed in 10% formol-saline solution for 24 h, processed, embedded in paraffin to prepare 5µm sections and stained with hematoxylin and eosin to compare the thickness of the epithelial cells by counting the layers of epithelial mucosa of the three groups and to detect the different histopathological changes .The second part was used for the immuno-histochemical study. Immuno-staining required antigen retrieval by heating the tissue sections in citrate buffer pH 6.0 in a microwave for two minutes followed by cooling at room temperature. Then they were incubated with the ready formed primary antibody for one hour [16] .The following primary antibodies were used: monoclonal antibody 6H2.1, anti-estrogen receptor, clone:

SP1, which recognizes ERα(the positive control is breast ductal carcinoma) and monoclonal antibody 6H2.1, anti-ki 67, clone: SP6 (the positive control is the tonsil). Then, ultravision universal detection system was used to detect the immune reaction. This was formed of biotinylated anti-polyvalent secondary antibody, streptavidin peroxidase and DAB[17].The sections were then counterstained using Mayer's hematoxylin [18].For negative control sections, the same procedure was followed with the omission of incubation into the primary antibodies. All these materials were purchased from Thermo Scientific, Lab Vision-Fisher Scientific, and USA.

Morphometric and statistical analysis:

Specimens were examined using the Leica Qwin 500 LTD image analyzer computer system.

Numbers of strata of the endometrium, ten non overlapping fields at magnification x400 per slide from five slides of each animal, selected at random were subjected to morphometry for the number of strata in the endometrium. One-way analysis of variance ANOVA was performed to assess the differences between control and the treated groups. Differences in means among treated groups were tested by using Duncan's test where P value < 0.05 was considered statistically significant.

RESULTS

Histological observations:

Light microscopic examination of the control group showed normal thickness of uterine mucosa which was formed of simple columnar partially ciliated epithelium with underlying highly cellular connective tissue lamina propria (Fig.1 A). Uterine mucosa of the rats received the low dose of BPA revealed few small focal patches of abnormal mucosal cells, degeneration of the epithelial lining and few of the endometrial glands (Fig.1 B).The high dose of BPA exhibited increased cytoplasmic hydropic degeneration which appeared as vacuolation of the cytoplasm with foamy appearance and numerous pyknotic nuclei. BPA induced proliferative changes in adjacent or remote areas to these degenerative patches with increased number of strata. Although numbers of layersincreased, the epithelium appeared riddled and many cells in different strata, even the basal ones, showed areas of hydropic degeneration and vacuolations with numerous pyknotic nuclei. (Fig.1 C).

Immuno-histochemical results:

Immunohistochemical examination of uterus of control rat revealed normal expression of ERα (Fig.2A).In sections of the second group that received the low dose of BPA and the third group that received the high dose, moderate and marked decrease of ERα appeared respectively (Figs. 2 B & C).

Immuno-histochemisrty of the normal control sections of the endometrium using Ki-67, a primary antibody as an indicator for cellular proliferation showed positive epithelial cells (Fig.3 A). Uterine epithelium of the rat received the small dose of BPA demonstrated moderate increase in the number of positive epithelial cells (Fig. 3 B), while endometrium of rats received the high dose exhibited marked increase in the number of positive Ki-67 in both epithelial and stromal cells (Fig.3C).

Area percent of ERα significantly decreased in both BPA treated groups and was dose dependent compared to the control (Table 1). A significant increase of proliferating epithelial cells was detected in animals given low dose of BPA compared to the control one and accentuated in the high dose treated group (Table 2). Number of strata increased significantly only in high dose group compared to the control (Table 3). A significant negative correlation between the number of strata in the endometrium and the area percent of ERα per field was also noticed ($r = –0.734$, $p = 0.001$) (Fig. 4). However, a significant positive correlation between the number of strata in the endometrium and Ki-67 percentage of immunopositive cells was detected ($r = 0.805$, $p = 0.001$) (Fig. 5).

DISCUSSION

Exposure to endocrine disturbing (ED) chemicals during critical periods of development as prenatal or early postnatal period could result in adverse effects to wildlife and humans. They may influence growth, reproduction and development [19]. The obtained results indicated that BPA caused degenerative effects in endometrium of rats.

Kathryn *et al.* [20] and **Lazúrová & Lazúrová** [21] analyzed the potential role of BPA on many organs and tissues. They gave marking, but not conclusive evidences, on its role in inducing proliferative and precancerous changes up to even frank carcinoma, like ductal carcinoma of the mammary glands [20] testes and prostate carcinoma [21].

Wang *et al.* [22] showed that exposure to BPA both in the low and high doses exerted hydropic degeneration of the endometrium and pyknosis. With the high dose of BPA there were areas of reactive thickening. **Howdeshell *et al***. [23] published details of the effects on female offspring of mice exposed in the womb on days 11 to 17 of gestation. The BPA dose was 2.4μg per kg body weight, which is a similar dose to that which caused effects on the male offspring, and a level equivalent to that typically found in the environment. The study suggested that trans-placental exposure to low doses of BPA could bring early puberty in female pups. The workers concluded that prenatal exposure to a dose of BPA, comparable to levels found in the environment, altered postnatal growth rate and reproductive function in female mice

Markey and Co-workers [24] showed that in-utero exposure to low, environmentally relevant doses of BPA induced marked changes in the vaginal cells and changes in the reproductive cycle. **Ben and Steinmetz** [25] reported that BPA caused cell changes in rat uterus and vagina, and the alterations caused were almost identical with those produced by estradiol. **Steinmetz *et al***. [26] identified that the vagina appeared to be particularly sensitive to the estrogenic actions of BPA.

At the tissue level, the absolute diameter of the lamina propria of the endometrium was significantly decreased in the animals exposed in utero to 250 μg BPA/ kg per day, while the remaining compartments of the uterus showed a decrease that was not statistically significant [27].

At the organ level, **Yoshida *et al.*** [28] and **Daston *et al.*** [29] reported morphological changes in reproductive tract tissues after exposure to significantly higher doses of estrogenic substances. For example, neonatal exposure to 2 mg per pup per day (Postnatal days 1–5) has been shown to induce hypertrophy of luminal epithelial cells, a decrease in the number of endometrial glands, and disorganization of the stroma and muscle layer, associated with an overall decrease in the size of the uterus.

Estrogens stimulate uterine epithelium proliferation *in vivo* and play a critical role in uterine growth, epithelial morphogenesis, cyto-differentiation, and secretory activity. The role of estrogen receptors (ER) is studied as a probable key of how it induces proliferative changes in the uterus. ERα localizes both nuclear and intracytoplasmic [30].

The present findings showed that the immune expression of ERα markedly reduced after prenatal exposure to BPA. This degradation of the sites of ERαmay be due to the mechanism of producing the histological degeneration in the uterine epithelium. There was a decrease of cell receptors in the stroma similar to that of the epithelium. A previous work claimed that, even at very low levels, xeno-oestrogens, being oestrogenic ED which can cause toxicological effects by binding hormone receptors, mimicking hormones or blocking their activities [31].

The mechanisms underlying the down regulation of ERa expression may antagonize the action of endogenous estrogens, because there are differences in the existence of tissue-specific ER co activators [32,33]. BPA being an ED chemical with weak oestrogenic activity potentiates playing similar role as potent estrogenic substances.

Antigen Ki-67 is a nuclear protein that is associated with cellular proliferation [34]. This means that these areas were subjected to kinds of unusual stresses which induced proliferative changes hampered by degenerative ones.Since Ki-67 was expressed in all proliferating cells, including normal and tumor cells, it was used as an excellent marker of the cell proliferation [35,36]to trace these proliferative changes, immune expression of Ki-67 was conducted. It showed increased proliferation of cells to compensate for accelerated degeneration that was induced by BPA. This may be due to mutations of the genes encoding transcription and translation processes during mitosis.

Conclusions and recommendations:

Human and wildlife exposure to BPA should be eliminated. Therefore releases to the environment should be prevented. This is because even a relatively low level can cause effects. Governments should provide information to mothers on the hazards associated with BPA and providing the available alternatives to polycarbonate feeding bottles for babies. Human exposure to BPA compounds should be eliminated. Therefore, the use of these substances in food packaging materials and in the manufacture of substances liable to come into contact with food and drink should be phased out.

REFERENCES

1. **Hoekstra EJ and Simoneau C (2013):** Release of bisphenol A from polycarbonate. Crit. Rev. Food Sci.Nutr., 53(4):386-402.
2. **Furuya M, Adachi K, Kuwahara S, Ogawa K and Tsukamoto Y (2006):** Inhibition of male chick phenotypes and spermatogenesis by bisphenol A. Life Sci., 78: 1767-1776.
3. **Wisniewski P, Romano R, Kizys M, Oliveira K, Kasamatsu T, Giannocco G and Chiamolera M (2015):** Adult exposure to bisphenol A (BPA) in Wistar rats reduces sperm quality with disruption of the hypothalamic-pituitary-testicular axis. Toxicology, 2:1-9.
4. **Lahnsteiner F, Berger B, Kletzl M and Weismann T (2005):** Effect of bisphenolA on maturation and quality of semen and eggs in the brown trout. Toxicology, 75: 213-224.
5. **Aarab N, Lemaire-Gony S, Unruh E, Hansen P, Larsen B, Andersen O and Narbonne J (2006):** Preliminary study of responses in mussel (*Mytilusedilus*) exposed to bisphenolA, diallyl phthalate and tetrabromodiphenylether. Aquat. Toxicol., 78: S86-592.
6. **Ortiz-Zarragoitia M and Cajaraville MP (2006):** Biomarkers of exposure and reproduction-related effects in mussels exposed to endocrine disruptors. Arch. Environ. Contam.Toxicol., 50: 361-369.
7. **Tsutsui T, Tamura Y, Yagi E, Hasegawa K, Takahashi M, Maizumi N, Yamaguchi F and Barrett JC (1998):**Bisphenol-A induces cellular transformation, aneuploidy and DNA adduct formation in cultured Syrian hamster embryo cells. Int. J. Cancer, 75: 290-294.
8. **Francesco D, Stefania P and Francesca F (2002):** Effects of perinatal exposure to bisphenolA on play behavior of female and male juvenile rats**.** Environ Health Perspect., 110: 403–407.
9. **Honma S, Suzuki A, Buchanan DL, KatsuY,and Iguchi T (2002):** Low dose effect of in utero exposure to bisphenol A and diethylstilbestrol on female mouse reproduction. Reprod. Toxicol., 16: 117–122.
10. **Birnbaum LS and Fenton SE (2003):** Cancer and developmental exposure to endocrine disruptors. Environmental Health Perspectives, 111: 389–394.
11. **Adriani W, Seta D, Dessi F, Farabollini F and Laviola G (2003):** Altered profiles of spontaneous novelty seeking, impulsive behavior, and response to D-amphetamine in rats perinatally exposed to bisphenol A. Environmental Health Perspectives, 111: 395–401.
12. **Spearow J, Doemeny P, Sera R, Leffler R and Barkley M (1999):** Genetic variation in susceptibility to endocrine disruption by estrogen in mice. Science, 285: 1259 – 1261.
13. **Varayoud J, Ramos J, Bosquiazzo V, Lower M, Muñoz-de-Toro M and Luque EH (2011):** Neonatal exposure to bisphenol A alters rat uterine implantation-associated gene expression and reduces the number of implantation sites. Endocrinology, 152: 1101–1111.
14. **Schönfelder G, Flick B, Talsness C and Paul M andChahoud I (2002):** In utero exposure to low doses of bisphenolA lead to long-term deleterious effects in the vagina. Neoplasia, 4: 98–102.
15. **Goldman T, Murr A and Cooper R (2007):** The rodent estrus cycle characterization of vaginal cytology and its utility in toxicological studies. Birth Defects Res., 80: 84–97.
16. **Bancroft J and Cook H (1994):** Manual of Histological Techniques and Their Diagnostic

Applications.2nd ed., W.B. Saunders Company,London, pp: 263-325.

17. **Ricardo B(2009):** Immunostaining Protocols for Flow Cytometric Analysis of Adherent Cells. R&D Systems.Pbl. Inc. Minneapolis, United States, p.11.
18. **Godwin A (2011):** Histochemical uses Of haematoxylin - A Review. Nigeria JPCS, 1: 24-34.
19. **Loder N (2000):** Royal society warns on hormone disrupters. Nature, 406: 4–15.
20. **Kathryn C, Wendy N, Kevin E, Steven L, Charles E, Patricia A, Catherine A and Carmen J (2014):**Bisphenol A exposure alters developmental gene expression in the fetal Rhesus Macaque uterus. PLoS. One, 9(1): 85894-85904.
21. **Lazúrová Z and Lazúrová I (2013):** The environmental estrogen bisphenol A and its effects on the human organism. VnitrLek., 59: 466–471.
22. **Wang H, Eriksson H and Sahlin L (2000):** Estrogen receptors alpha and beta in the female reproductive tract of the rat during the estrous cycle. Biol.Reprod., 63: 1331–1340.
23. **Howdeshell K, Hotchkiss A, Thayer K and Vandenbergh J (1999):** Exposure to BPAadvances puberty. Nature, 401: 763-764.
24. **Markey C, Michaelson C, Veson E, Sonnerschein C and Soto A (1999):** Estrogenic effects of bisphenol A in mice following in utero and post-natal exposure presented at environmental hormones: Past, Present, Future, Tulane University, pp: 18-20.
25. **Ben J and Steinmetz R (1998):** Xenoestrogens, the emerging story of bisphenol A. Trends Endocrinol.Metab., 9(3):124-128.
26. **Steinmetz R, Mitchner N, Grant A, Allen D and Ben-Jonathan, N. (1998):** The xenoestrogen BPA induces growth, differentiation, and c-fos gene expression in the female reproductive tract. Endocrinology, 139:2741-2747.
27. **Gilbert S, Karin F, Martin P and Ibrahim C (2004):**Developmental effects of prenatal exposure to bisphenol A on the uterus of rat offspring**.** Neoplasia, 6(5): 584–594.
28. **Yoshida A, Newbold RR and Dixon D (1999):** Effects of neonatal diethylstilbestrol (DES) exposure on morphology and growth patterns of endometrial epithelial cells in CD-1 mice. Toxicol.Pathol., 27:325– 333.
29. **Daston G, Cook J, and Kavlock R (2003):** Uncertainties for endocrine disrupters: our view on progress. Toxicol. Sci., 74: 245 – 252.
30. **Raso MG, Behrens C, Herynk MH, Liu S, Prudkin L, Ozburn NC, Woods DM, Tang X, Moran C, Lee JJ and Wistuba II (2009):** Immunohistochemical expression of estrogen and progesterone receptors identifies a subset of NSCLCs and correlates with EGFR mutation. Clin. Cancer Res., 15: 5359–5368.
31. **Dang V, Choi K and Jeung E (2009):** Estrogen receptors are involved in xenoestrogen induction of growth hormone in the rat pituitary gland. J.Reprod. Dev., 55: 206–213.
32. **Abargues M, Ferrer J, Bouzas A and Seco A (2013):** Estrogen receptors are involved in xenoestrogen induction of growth hormone in the rat pituitary gland. J .Reprod. Dev., 55: 206–213.
33. **Suzuki A, Sugihara A, Uchida K, Sato T, Ohta Y, Watanabe H and Iguchi T (2002):** Developmental effects of perinatal exposure to bisphenol-A and diethylstilbestrol on reproductive organs in female mice. Reprod. Toxicol., 16: 107–116.
34. **Fung A, Cohen C, Kavuri S, Lawson D and Gao X (2013):** Phosphohistone h3 and Ki-67 labeling indices in cytologic specimens from well-differentiated neuroendocrine tumors of the gastrointestinal tract and pancreas: a comparative analysis using automated image cytometry. Acta.Cytol., 57: 501–508.
35. **Stygar D, Muravitskaya N, Eriksson B, Eriksson H and Sahlin L (2003):** Effects of SERM (selective oestrogen receptor modulator) treatment on growth and proliferation in the rat uterus. Reprod.Biol.Endocrinol., 1: 40–48
36. **Nnene IO, Nieto JJ and Crow JC (2004):** Cell cycle and apoptotic proteins in relation to ovarian epithelial morphology. Gynecol.Oncol., 92: 247–251.

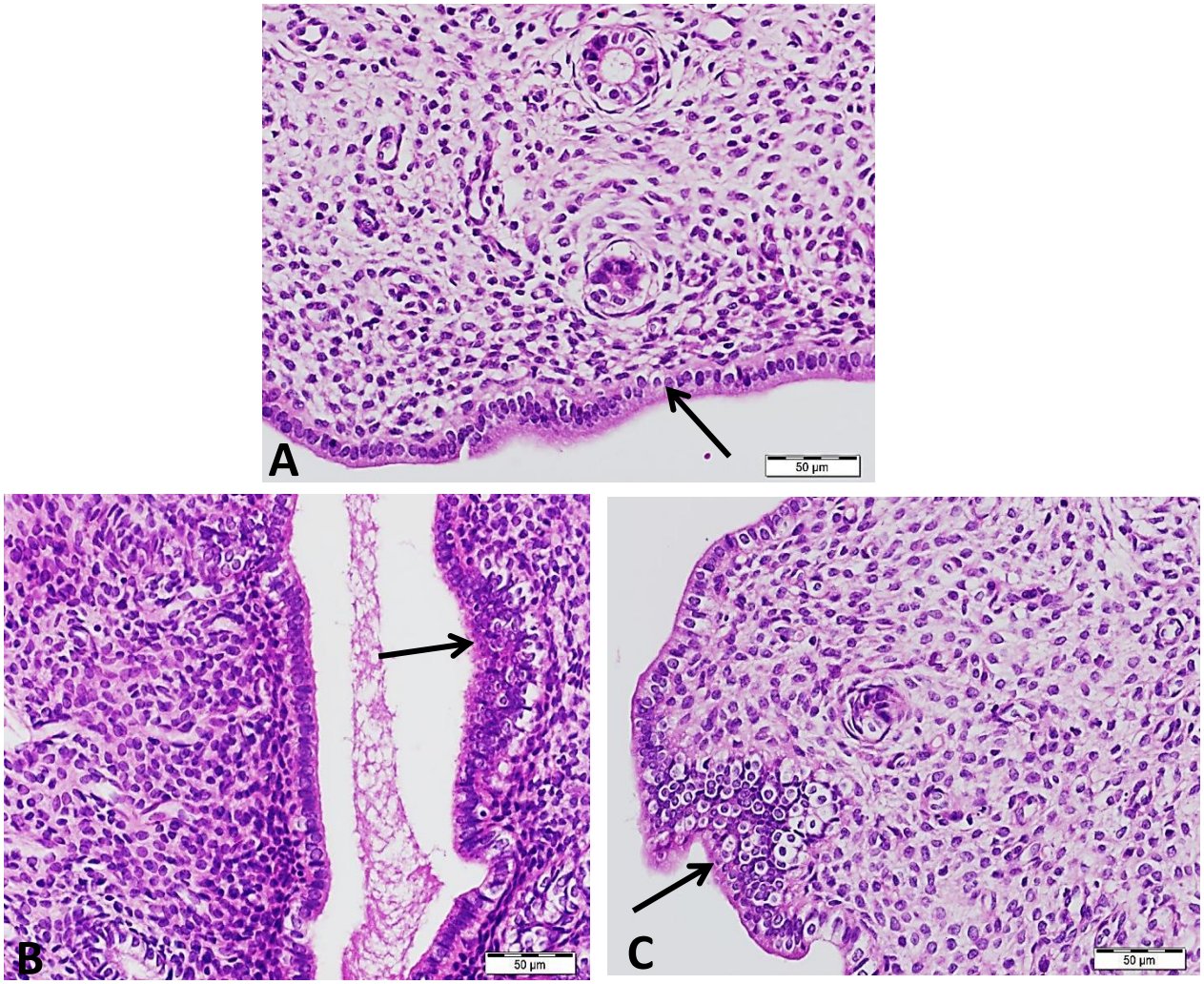

Fig.1. Light photomicrographs of sections of uteri stained with hematoxylin and eosin showing (A) G1, control endometrium with normal thickness of epithelial mucosal layer formed of simple columnar partially ciliated epithelium (arrow) with underlying highly cellular connective tissue lamina propria. (B) Uterine mucosa of rat received the low dose of BPA (G2) showing few small focal patches of abnormal increased numbers mucosal cells (arrow) with few endometrial glands. (C) Uterine mucosa of rat received the high dose of BPA (G3) showing many large areas of epithelial proliferation (arrow), most of their cells are vacuolated and their nuclei showing pyknosis.

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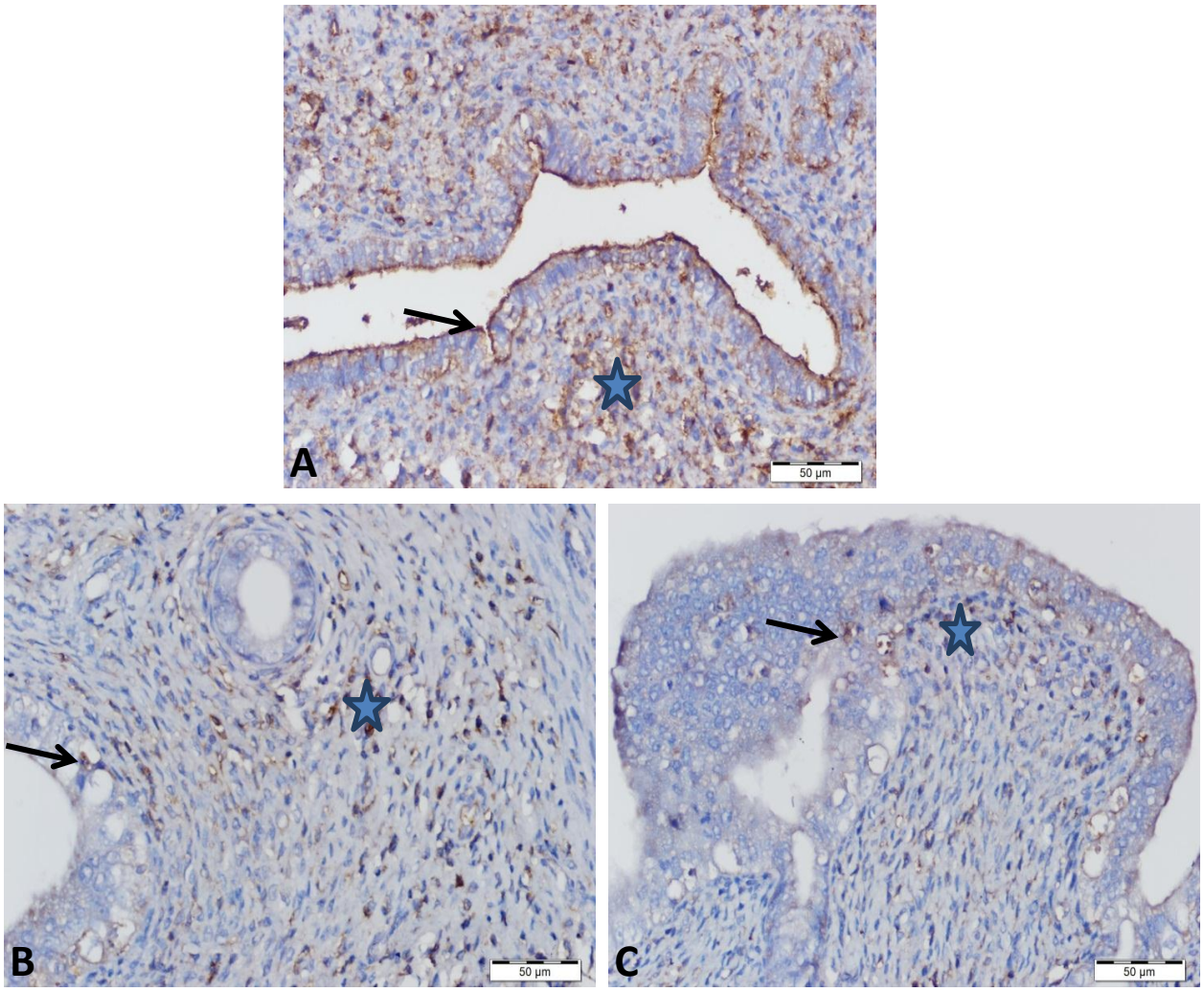

Fig. 2. Light photomicrographs of sections of uteri immuno-stained with ERα, showing: (A) G1, control endometrium with normal immuno-positive cells in the epithelium (arrow) and stroma (star). (B) G2, endometrium of rat received the low dose of BPA showing moderate decreased expression of ERα in the epithelium (arrow) and stroma (star). (C) G3, endometrium of rat received the high dose of BPA showing a marked decrease of ERα immune expression in both epithelium (arrow) and stroma (star). (X 400)

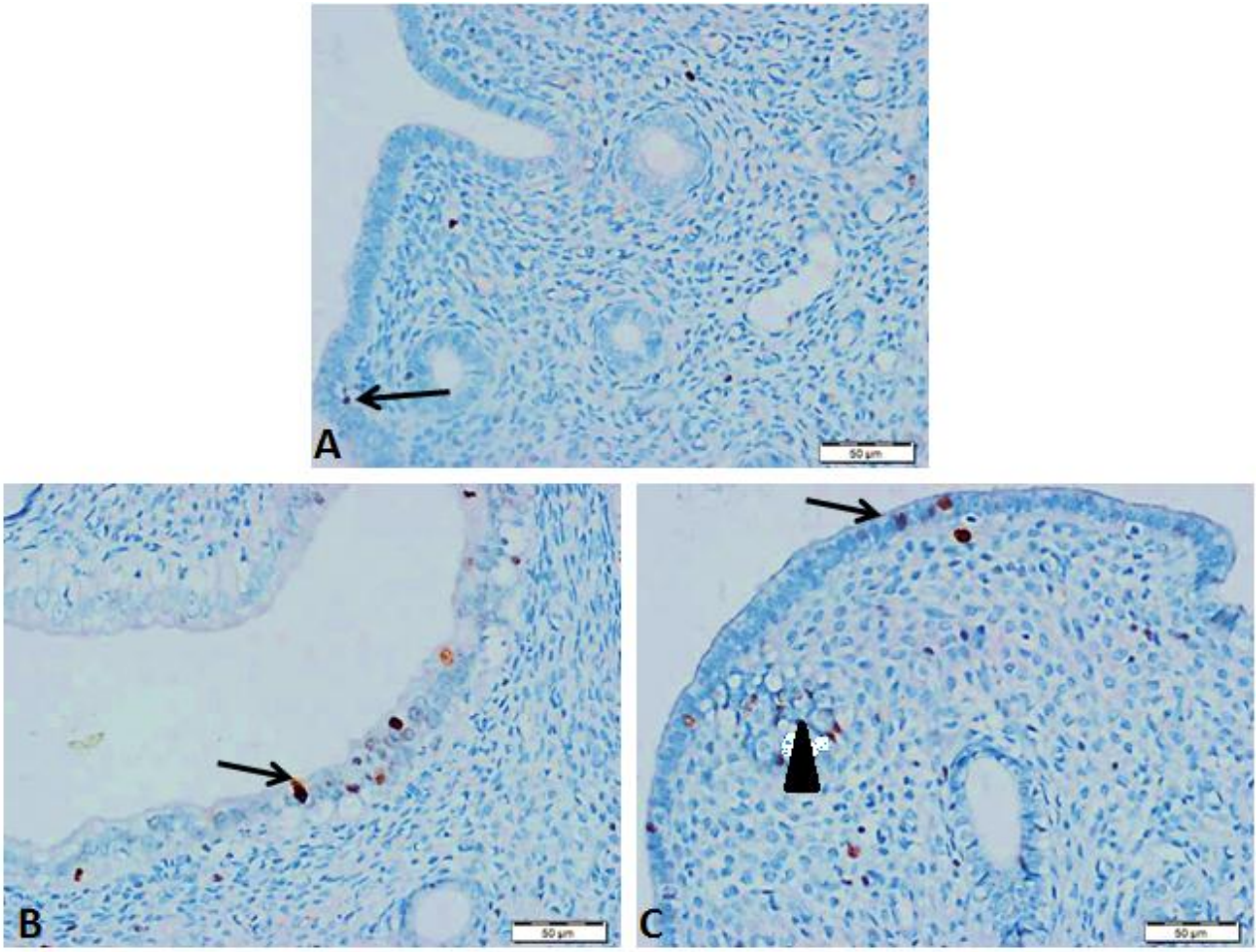

Fig. 3. Light photomicrographs of sections of uteri showing expression of Ki-67 as a proliferative factor indicator showing: (A) G1, normal control endometrium with normal epithelial cells (arrow). (B) G2, endometrium of rat received the low dose of BPA showing moderate increase of positive epithelial cells (arrow). (C) G3, endometrium of rat received the high dose of BPA showing marked increase of number of positive epithelial cells (arrow) and stroma (arrow head). (X 400)

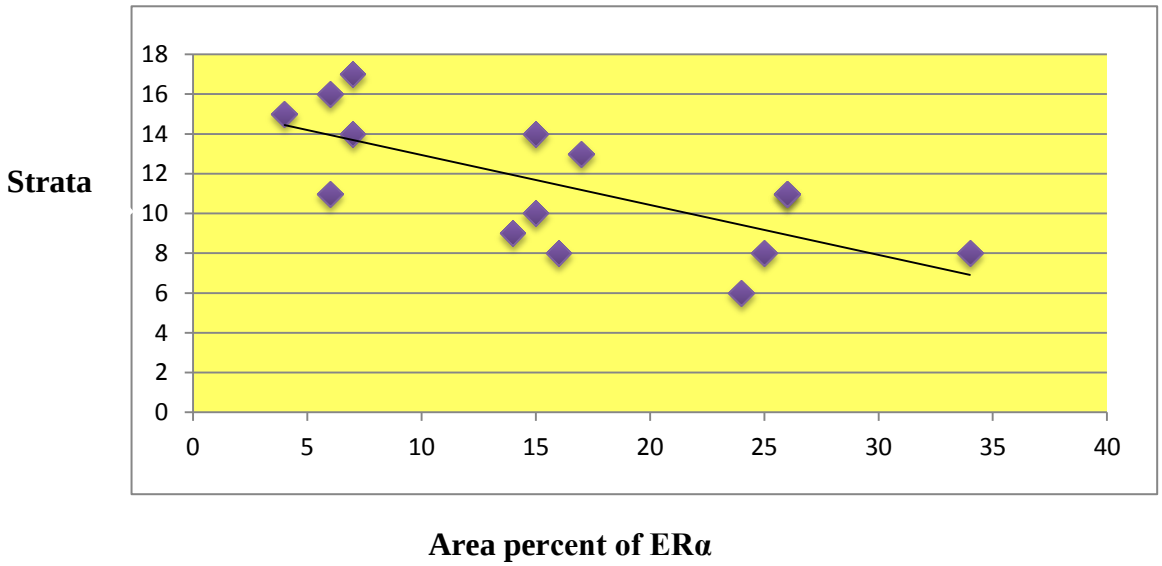

Fig. 4. Correlation between numbers of strata of the endometrium and area percent estrogen receptors α (ERα)

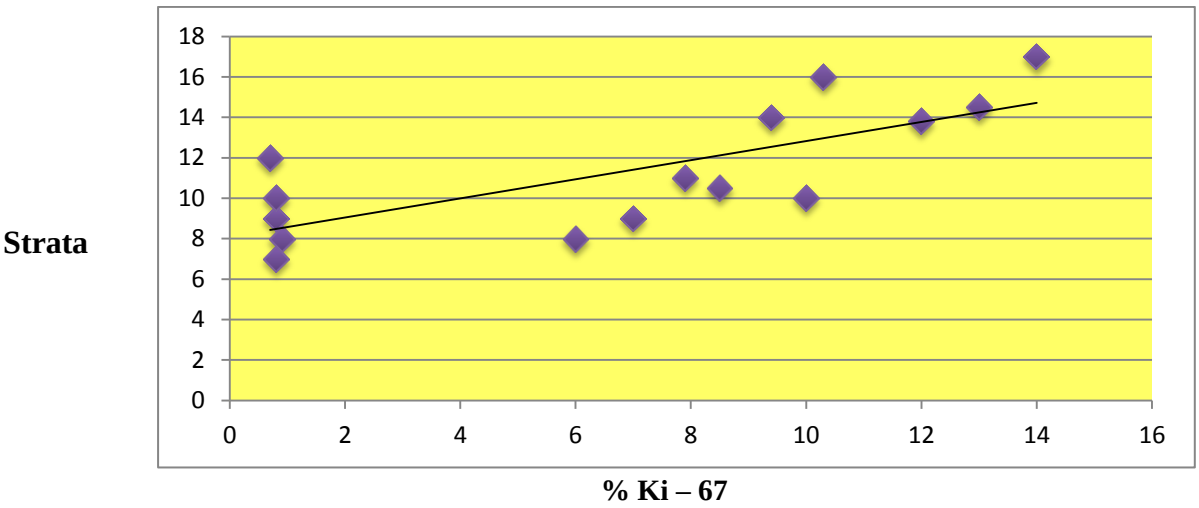

Fig. 5. Correlation between numbers of strata of the endometrium and proliferation marker (Ki-67) percentage of immuopositive cells

Table 1: area percent of estrogen receptors a (ERα) immunopositive cells per field

	Mean ± SD	**P**
Group 1	27.09 ± 6.72	-
Group 2	18.48 ± 4.83	0.29*
Group 3	8.6 ± 4.95	0.002*

*P value < 0.05 significant compared to group 1

Table 2: percentage of proliferation marker Ki-67 immunopositive cells per field

	Mean ± SD	**P**
Group 1	0.66 ± 0.09	-
Group 2	7.89 ± 1.36	0.001*
Group 3	12.67 ± 2.08	0.001*

*P value < 0.05 significant compared to group 1

Table 3: number of strata of the endometrium.

	Mean ± SD	**P**
Group 1	10.2 ± 1.92	-
Group 2	12.4 ± 2.20	0.286
Group 3	14.6 ± 2.11	0.005*

*P value < 0.05 significant compared to group 1