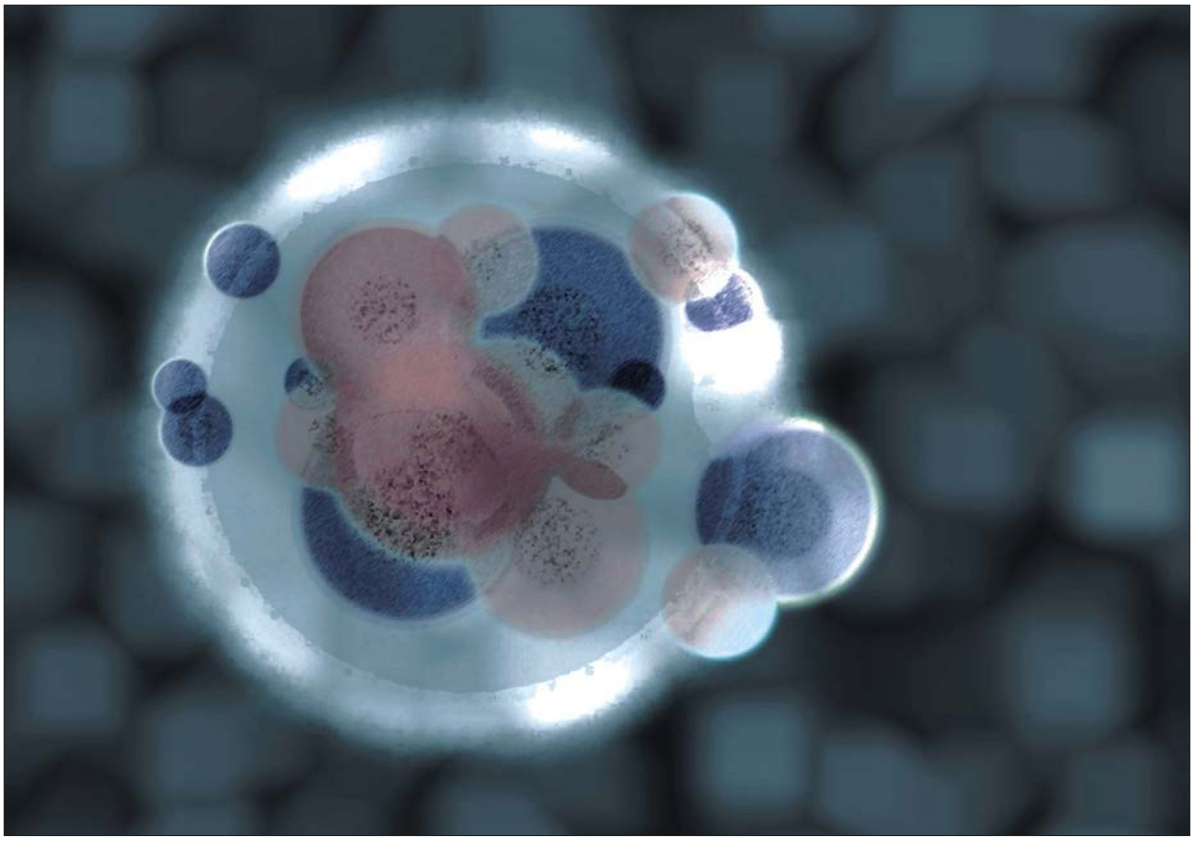

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The Effects of Clomiphene Citrate Administration in Ovariectomised Rats: An Ultrastructural Study

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Abstract: In this study, we aimed to investigate Clomiphene Citrate (CC) on vagen of adult Ovariectomised rats. Transmission electron microscopy and light microscopy have been used to study changes to the vaginal epithelium induced by C administered to Ovariectomised rats. The study was performed in Department of Medical Science Application and Research Centre of Dicle University, DUSAM-Turkey in 2001. Ten female, Wistar Albino rats were used and were divided into three groups. At the beginning and end of the administrations, body weight of animals was determined. Animals were sacrificed 35 days after ovariectomised and vaginal tissue was examined by transmission electron microscopy and light microscopy. We found that, the body weight was higher in the ovariectomized rats than the control rats. Further, body weight increased after clomiphene citrate therapy. It was found that clomiphene citrate treatment produced ultra structural surface features some of which were similar to those seen with hormone treatments and others unique to clomiphene alone. In conclusion, antiestrogen clomiphene citrate administration affects vagina epithelium and lead to increasing body weight.

Key words: Clomiphene citrate, vagina, ultrastructural changes, rat

INTRODUCTION

Infertility is observed in approximately 10 to 15% of couples of reproductive age (Speroff *et al*., 1999). Ovulation disorders account for about 30 to 40% of female infertility and about 20% of infertility in couples. Several treatment modalities have been suggested for women with ovulation disorders, but the first-line regimen for medical induction of ovulation is Clomiphene Citrate (CC) for most patients (Horstein and Schust, 1996). Anti-estrogenic drugs, such as clomiphene citrate, are the first-line therapy for ovulation induction in these patients and are capable of producing ovulation in 70-75% of cases. Satisfactory response is seen in 70% of patients when clomiphene citrate is given at a dosage of 50-100 mg day^{-1} (Sohrabvand *et al*., 2006).

Reproductive function is controlled by a very sophisticated system composed by the perfect synchronization of neural and endocrinological functions (Genazzani, 2005). Hormone replacement strategies have limited benefit because they do not promote recovery from these allostatic endocrine adjustments in the Hypothalamus-Pituitary-Ovarian (HPO) axis. Although the menstrual pattern can be restored with exogenous administration of estrogen/progesterons, the long term deleterious consequences of stress-induced anovulation may lead to increased risk of cardiovascular disease, osteoporosis, depression and other psychiatricconditions (Berga and Loucks, 2005). Clomiphene Citrate (CC) has been applied as the first-line treatment in anovulatory women since the 1960s, due to its low cost and minor side-effects or complications (Van Santbrink *et al*., 2005). It is recognized that CC has partial antagonist/partial agonist properties and it dominant effect on the HPO axis is related to its antiestrogenic properties (Fiad *et al*., 1998). Dehbashi *et al*. (2006) found that treatment with CC is associated with higher rates of pregnancy if started early (days 1-5) in the menstrual cycle. Although CC induced ovulation rates are between 80 and 85% conception rates are only around 40% (Clark and Markaverich, 1982; Bilijan *et al*., 1999; Dehbashi *et al*., 2006). This could be due to negative effects of CC on ocyte or granulose cells, or because of prolonged antiestrogenic effects on the endometrium and cervical

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mucosa. These negative effects are augmented by the relatively long half life of CC, which is known to be 5 days. If treatment is started late in the cycle, those negative effects are more likely to extend into the sensitive peri-implantation period (March, 1992).

Clomiphene, an established clinical agent for the induction of ovulation in sub fertile women, is a substituted triphenylethylene that is considered to be an antiestrogen, based on ability to antagonize uterine growth and vaginal cornification induced by estrogen in immature rodents (Massai *et al*., 1993; Jordan, 1984; Borges *et al*., 2007).

Chemically, CC (like tamoxifen) is a nonsteroidal triphenylethylene derivative that exhibits both estrogen agonist and antagonist properties. In general, estrogen agonist properties are manifest only when endogenous estrogen levels are extremely low. Otherwise, CC acts solely as a competitive estrogen antagonist. Clomiphene citrate is cleared through the liver and excreted in stool. About 85% of an administered dose is eliminated after approximately 6 days, although traces may remain in the circulation for much longer (Clark and Markaverich, 1982).

It is obvious to anyone who has occasion to study the estrogenically stimulated vagina that there must be a rapid and continuing production of cells in the basal cell layer to replace those that are shed from the surface in such large numbers (Wu and Winkel, 1989).

The aim of this study was to investigate whether the timing of administration of CC affects vaginal thickness and body weight in ovariectomized rat model. This study therefore uses transmission electron microscopy to examine the effects of CC on the surface ultra structure of the luminal epithelial cells.

MATERIALS AND METHODS

Three-month-old (14 weeks old), virgin Wistar-Albino female rats (were obtained from the Department of Medical Science Application and Research Centre of Dicle University, Turkey in 2001 (DÜSAM), weighing about 200-220 g, were feed with standard pellet food and water *ad libitum* during the study. For adaptation, each rat was placed in an individual cage in the experimental period. All animals received human care according to criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and Published by the National Institutes of Health. The ambient temperature (22°C) and relative humidity (45%) were maintained on a 12 h light/dark cycle throughout the experiments.

Bilateral ovariectomies were performed, except for sham-operated (SHAM) controls and maintained without treatment for 35 days, this rats were given an intraperitoneal injection with 0.1 mL of carrier A alone (1:3 mixture of 100% ethanol and 0.9% saline W/V (Boots CO., Australia) each day for 3 days. Carrier An injection was given in order to create the stress environment as in the two other animal groups. One group of 2 ovary-intact rats was killed on the day of surgery to provide initial results. Surgical ovariectomy was performed to group 2 and group 3 (n = 8)

Ovariectomy: In brief, group 2 and 3 rats were food-deprived prior to surgery. For the ovariectomies, the rats were anesthetized by intraperitoneal injection of Ketamine HCl (50 mg kg^{-1} b.wt. Parke-Davis) and xylazine 2% (100 mg kg^{-1} b.wt. Rompun-Bayer). Bilateral ovariectomies were done in 8 animals using a dorsal approach in a sterile surgical theatre. After bilateral ovariectomy, the rats were allowed to recover for 3-4 weeks before being treated.

The second group used OVX (ovariectomized) groups {without clomiphene citrate}. Then, OVX rats were subcutaneously injected with equivalent amount 0.1 mL of a 1:3 mixture of benzyl alcohol and peanut oil, each day for 3 days.

Group 3 is designated as sham-operated, bilaterally ovariectomized with clomiphene citrate groups. Animals in group 3 were given intraperitoneal injections of 0.1 mL carrier containing 100 mg CC (44-55% en-zu mixture, Sigma Chemical Co., St. Louis, Mo.) each day for 3 days.

After approximately four-weeks of ovariectomy, the animals were anaesthetized and killed by cardiac exsanguinations. Pelvic region was seen to be far too much fatty at autopsy. Vaginae were removed, pinned out on dental wax to their *in vivo* length and placed immediately in a 2.5% phosthate-buffered glutaraldehyde solution at pH 7.4 for 4 h. Tissue pieces were chosen at random, then split along the long axis into 2 or 3 pieces and placed in fresh fixative for a further 40 min, post fixation was performed in 2% osmium tetroxide and washed in three changes of phosphate buffer, pH 7.4 dehydrated in graded alcohols. Sample tissues were embedded Araldyte-Cy 212. Semi thin sections were stained with toludine blue. Thin sections were stained with uranyl acetate and lead citrate. The specimens were examined and photographed using Karl Zeiss EM-900 transmission electron microscope.

For light microscope, the vagina was removed from each rat at autopsy. Next, vagina was immersed in 4% Para formaldehyde for 24 h at 4°C for fixation. They were then transferred into the tap water until further processing. The

vagina tissue was processed into paraffin blocks, through successive concentration of ethanol and xylene. Five micron sections were cut using a Leica Supercut 2065 and the sections were stained with hematoxylin-eosin and Mallory azan.

Statistical analysis: Data are expressed as mean±SEM. Statistics were calculated using Minitab. The analysis of variance (ANOVA) test followed by Fisher s was used to compare the differences between groups (Neter *et al*., 1982).

RESULTS

Body weight: Comparison of the live weight means of control group with the other animals after the second month (postovariectomy) was found to be important ($p<0.01$) (Table 1).

In this trial, paired t-test was performed in order to elucidate whether the administration of the drug was effective in those animals or not and it was determined that administration of the drug was effective on body weight ($p<0.05$) (Table 1).

Light microscopy results: Vaginal appearance of sham-operated-controls group (group 1) was normal. The lumen of the vagina was lined by a thick stratified squamous non-keratinized epithelium (Fig. 1). In the vaginal epithelium of ovariectomised groups a typical atrophied epithelium appearance was observed (Fig. 2). The vaginal epithelium of the groups treated with CC had an appearance of thickness and cornification due to remarkable hyperplasia and epithelial down growth (Fig. 3).

In the length of luminal epithelium in CC group however a marked increase as well as metaplastic alterations that turned into stratified columnar epithelium was observed.

The nuclear to cytoplasmic ratio appeared low and nucleoli were prominent. Mitotic figures were found in basal and intermediate layers. There was no cytoplasmic vacuolation present in cells of the surface layer with 100 mg CC treatment but there was a defined line tat separated the surface cells from the layer immediately underneath.

Electron microscopy results: In the vaginal epithelium of ovariectomised groups have thin and irregular basal membrane (Fig. 4, 5).

After treatment with CC numerous variation are observed. Nuclei with heterochromatin, mitochondria were swollen and the rough endoplasmic reticulum was very

Table 1: The comparison of the body weights in all groups

	Control n = 2	OVX n = 4	OVX/CC n = 4
Body weight (g):	207±2.4*	228±3.5*	250±0.0*

*: Statistically significant difference between all groups $p<0.05$

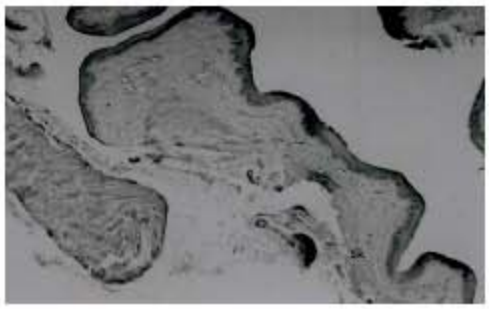

Fig. 1: Vaginal epithelium of sham-operated controls group animals showing, stratified squamous non-keratinized epithelium (~), sk(sirkuler muscle) (Hematoxylin-Eozin X100)

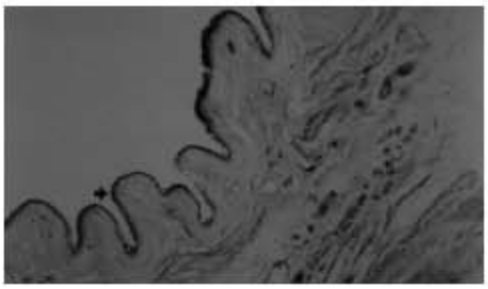

Fig. 2: In the vaginal epithelium of ovariectomized group animals showing typical atrophied appearance of the epithelium, nonkeratized (~) (Alcian-Blue/Methylene-Blue X100)

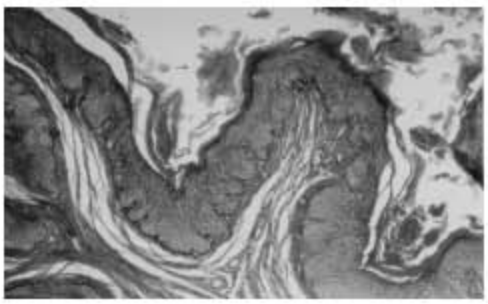

Fig. 3: The vaginal epithelium of the groups treated with clomiphene showing, increased in the thickness of the epithelium layer and keratized epithelium (~) (Mallory-Azan X200)

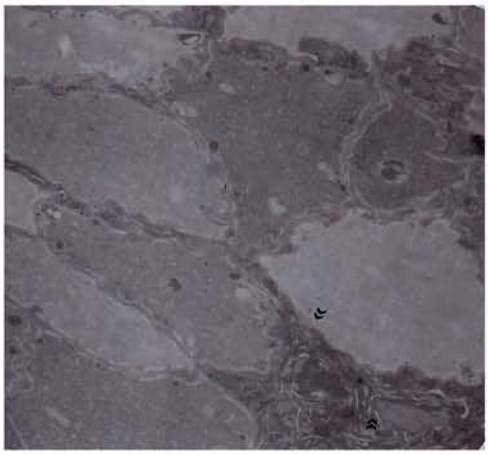

Fig. 4a: The ultrastructure appearance of the vaginal epithelium 4 weeks after ovariectomy, it is observed that basal lamina has become thinner and taken an irregular shape (») (Uranyl acetate-Lead citrate X3000)

Fig. 4b: In the higher enlargement of the vaginal epithelium of ovariectomized group, thinning an irregular structure are observed in the basal lamina (>) and some cells lost their nuclei, taking a picnotic shape (Uranyl acetate-Lead citrate X7000)

distinct. Some tonofibril distinguishable and epitel cells cytoplasm have a great deal of lipid granule. After treatment with CC luminal epithelium cells basal membrane are regular.

It is our purpose here to present some observation on the kinetics of the vaginal cell population of rats under conditions of constant CC stimulation. These data will, we hope prove useful in improving our understanding of vaginal physiology and form the

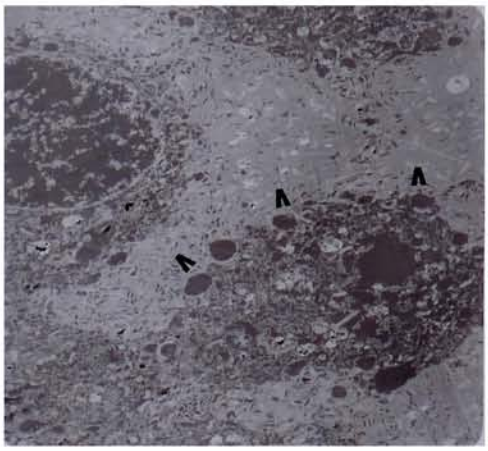

Fig. 5a: The ultrastructural appearance of the vaginal epithelium of rats exposed to clomiphene treatment following the ovariectomy. It is observed that apical cells have quasi a normal appearance and that there are plenty of lipid and glycogen inclusions (>) (Uranyl acetate-Lead citrate X3000)

Fig. 5b: In the higher enlargement of the vaginal epithelium of this group, the observation of plenty of lipid and glycogen inclusions around the nuclei of the vaginal cells was also striking (>) (Uranyl acetate-Lead citrate X7000)

basis for further investigation of the mechanism of action of various hormones as they affect this target cell population.

DISCUSSION

It is well known that vaginal tissues in different animal species, including human vaginal tissue, respond

to the action of endogenous or exogenous estrogens. Estrogen Receptors (ER) have been reported in vaginal tissues of various species, e.g., rabbit, mouse and human. However no information is available at present on the existence of ER and Progesterone Receptors (PR) in the vagina during fetal life (Nguyen and Grambiagı, 1986). In recent years, a correlation has become apparent between neonatal estrogen exposure and reproductive abnormalities.

Exogenous estrogens have teratogenıc and carcinogenic effects on the developing female genital tract of both laboratory animals and humans. Steroidal and nonsteroidal estrogens such as diethylstilbestrol (DES) elitic adverse effects. Certain triphenylethylene compounds, such as clomiphene, tamoxifen and nafoxidine are hormonally active and may exhibit antiestrogenic activity. Both clomiphene and tamoxifen are currently administered therapeutically to women. Clomiphene is administered as a fertility drug to induce ovulation in fertilite women (Cunha *et al*., 1987). It is apart of the dogma of steroid hormone action that it binds specially to its receptors intracellularly and this ligand-receptor complex alters the transcriptional activity of the hormone responsive genes. Antiestrogens inhibit the action of estrogen on its target organs by binding to the estrogen receptor, therefore, antiestrogens are commonly used for the therapy of estrogen receptor-positive human breast cancer and for the induction of ovulation (Terry *et al*., 1992).

Sex steroids administered during a critical neonatal period of development have marked lasting effects on reproductive function. Female rats treated in such a manner are infertilite due to an abscence of ovulation and corpora lutea formation, vaginal semears are persistently cornified and mating behavior is absent. Evidence has been presented to show that the acylic male pattern of gonadotropin secretion results from permanent damage to hypothalamic centers normally responsive to steroid feedback (Peckham and Klekhofer, 1962).

Kronenberg and Clark (1985) reported that the vagina is lined by a specialised epithelium where proliferation and differentiation are dependent upon the levels of circulating hormones. When the hormonal status is manipulated by *in vivo* exogenous hormone or drug treatments, differential growth and cell modification can be monitered.

Barker and Walker (1969) showed that earlier work indicates that ostrogen has the ability to initiate cell division, cause proliferation and aventually keratinisation of the epithelium. While exogenous progesterone also has the ability to initiate cell division in the dormant vaginal epithelium it does not result in a significant thickening of the epithelium, but causes extensive tissue folding.

Ladinsky and Peckham (1965) claimed that the increase in epithelial height seen in ovariectomised animals given exogenous estrogen can be attributed to the ability of estrogen to activate a dormant cell population within the generative compartment and shorten the G_1 stage in the progenitor cell cycle.

Observations in the present study on vaginal epithelium from ovariectomised animals treated with CC show tat the drug have an estrogenic component of action. This is indicated by an increase in the number of cell layers and increased epithelial area, height and mitotic activity when compared with the untreated tissue.

Scanning electron microscopy examining shows that, the vaginal wall exhibited longitudinal folding and the flattened cells were arranged as a mosaic of overlapping sheets with well defined leading edges. Raised microridges covered the surface of the vaginal cells and displayed some remnants of bulb-tipped short microvilli (Parakkal, 1974).

The findings of this study on the hormonal effects of exogenous estrogen and progesterone on modification of the surface layer agree with and extend those of parakkal (Leavitt and Meismer, 1968). In comparison to the effects of these two hormones, the surface of CC treated epithelium exhibits a covering of short microvilli characteristic of progesterone. However, it also presents unique characteristic peculiar to CC, including areas of microvilli clumping to from rosettes and individually elongated microvilli.

Different authors have reported emphasis to date has been placed on the effects of anti-estrogens on the reproductive tract during the perinatal period (Clark and Mc Cormack, 1977; Forsberg and Kalland, 1981). These studies have revealed that early vaginal opening and cornification and the presence of adenosis-like lesions caused by CC are similar to those observed with neonatal exposure to estrogen. Adashi *et al*. (1980) reported thus it may be the estrogenic component of CC in combination with prolonged occupancy of estrogen receptors, long half-life and its own ability to modify cell appearance that causes the reproductive teratology observed in adult animals. When animals are given 100 mg CC there is an increase in the number of spinous layers accompanied by nuclear involution of the surface cells indication a stronger estrogenic effect. Thus it is evident that CC, at the doses used in this study. Strongly promotes hyperplasia of the vaginal epithelium as a retarded aestrogen, but this growth is accompanied y unusual cellular and surface modifications.

Structural similarity to estrogen allows CC to bind to Estrogen Receptors (ER) throughout the reproductive system. However, in contrast to estrogen, CC binds nuclear ER for an extended period of time and ultimately

depletes ER concentrations by interfering with the normal process of ER replenishment (Kerin *et al*., 1985). The drug's effectiveness in ovulation induction can be attributed to actions at the hypothalamic level. Depletion of hypothalamic ER prevents correct interpretation of circulating estrogen levels. Reduced levels of estrogen negative feedback trigger normal compensatory mechanisms that alter pulsatile hypothalamic GnRH secretion to stimulate increased pituitary gonadotropin release that, in turn, drives ovarian follicular activity. In ovulatory women, CC treatment increases GnRH pulse frequency (Kettel *et al*., 1993). In anovulatory women with polycystic ovary syndrome (PCOS) in whom the GnRH pulse frequency is already abnormally high, CC treatment increases pulse amplitude, but not frequency (Rebar *et al*., 1976). During CC treatment, levels of both LH and FSH rise, falling again after the typical 5-day course of therapy is completed (Reyes *et al*., 2000). In successful treatment cycles, one or more dominant follicles emerge and mature, generating a rising tide of E2 that ultimately triggers the midcycle LH surge and ovulation.

Chronic anovulation is a common neuroendocrine cause of infertility. Pharmacological management of such condition includes the use of Clomiphene Citrate, which is the most utilized of the ovulation inductors. Clinical efficacy of C provides ovulation rates of around 70%. Those patients who did not respond are candidates to receive gonadothrophin treatment, which will increase costs and the risk of ovarian hyperstimulation and multiple pregnancies. Because of that, new alternative treatments have been assayed, such as the concomitant use of dexametasone. This alternative has improved the response, but its use has been limited because the side effects of this treatment overcome their benefits. Another alternative is the use of prednisone throughout the menstrual cycle together with CC administered in pre-established days. This treatment is expected to improve the response without adding side effects (Wurfel *et al*., 1999).

We conclude that, clomiphene citrate is an antiestrogen which exhibited estrogen-like effects. Stimulate the development of multiple eggs for use with assisted reproductive technology, such as *in vitro* fertilization (IVF) or gamete intrafallopian transfer.

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