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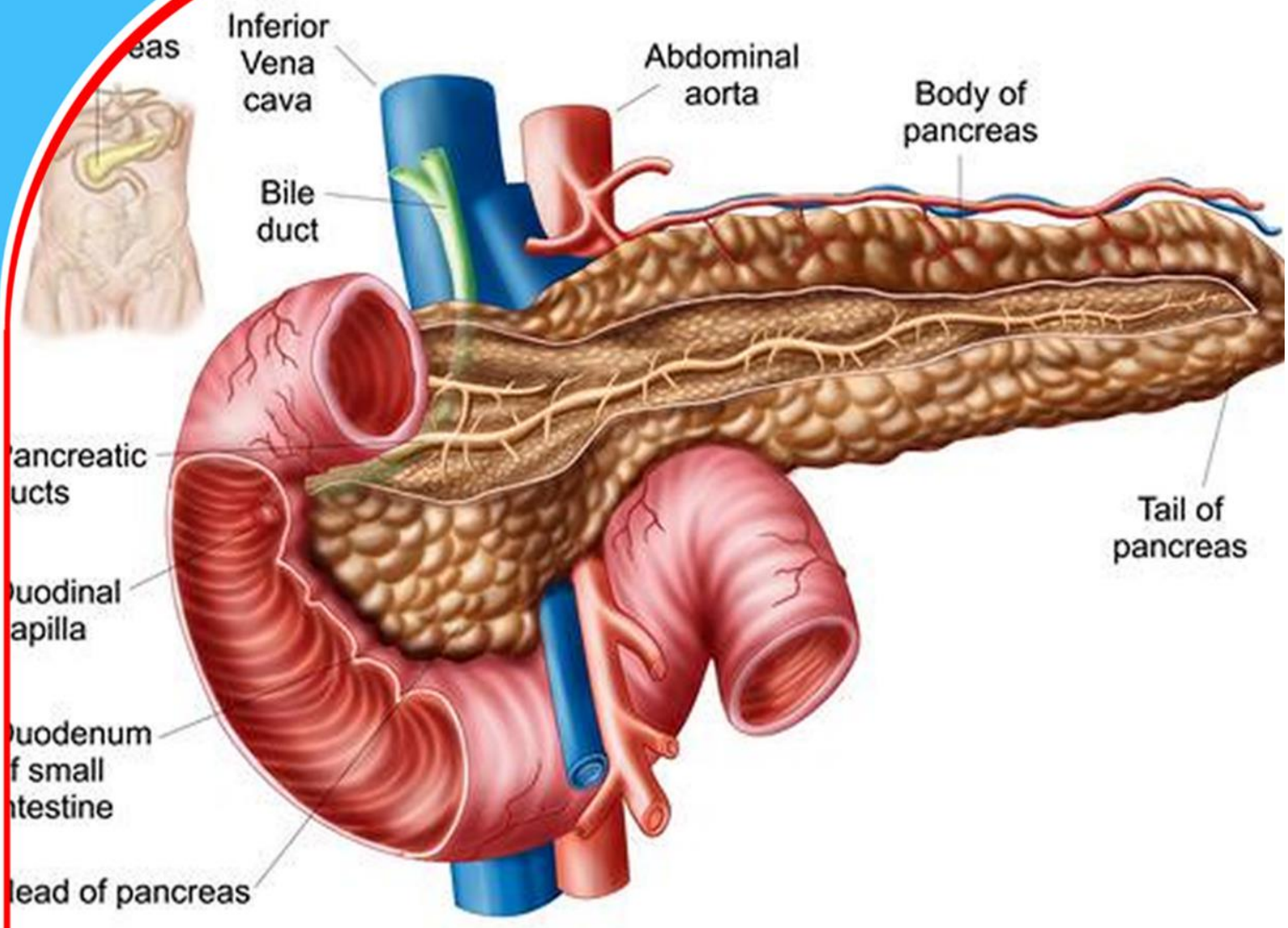

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Abstract

Purpose: Clomiphene citrate (CC) is used in the management of infertile females. Estrus Cycle indices are good parameters for evaluating reproductive changes, while actual ova shed represents evidence of ovulation. This study is aimed at investigating how different doses of CC can affect the Estrus Cycle (EC), Estrus Cycle Ratio, (ECR), and ova shed (OS) in adult female rats.

Methodology: Twenty-five (50) female Sprague Dawley (SD) rats were divided into 5 groups. Group A was controlled while groups B, C, D, and E were treated groups. Group A received 0.5 ml of sterile water. Groups B, C, and D were given 0.2 mg/kg, 2 mg/kg, and 4 mg/kg respectively at diestrus. Group E was given 6mg/kg/day of CC dissolved in sterile water. The administration was done orally. A vaginal smear was evaluated for various estrus phases. ECR was calculated, and ova shed at the estrus phase was evaluated at autopsy. All data were presented as mean ± standard error of the mean (SEM). Statistical significance was taken at $P < 0.05$

Findings: There was a significant decrease ($p<0.05$) in the number of proestrus, estrus, metestrus, and diestrus in treated groups compared with control. ECR value in all treated groups also decreased significantly ($p<0.05$) when compared with control. This study further shows that all the animal groups (A-E) released ova but there was a significant decrease ($p<0.05$) in the number of ova released in groups B, C, D, and E against the control group.

Conclusion: CC decreases EC. It reduced ECR, hence prolonging luteogenesis. It decreases the number of eggs released. With a large dose and prolonged use, eggs were still released even when the ova released and counted were fewer compared with when this drug was not in use.

Recommendation: Infertile couples should copulate more at the luteal phase of the menstrual cycle when on CC to increase their chances of pregnancy. CC decreases the number of eggs released but makes the few eggs released stay longer for fertilization.

Keywords: *Estrus Cycle, Estrus Cycle Ratio, Clomiphene Citrate, Luteogenesis, Ova Shed.*

1.0 INTRODUCTION

Infertility is a common socioeconomic problem, and which study reported that it has a 9 to 18% prevalence in the general population (Aghajanova et al., 2016). Clomiphene citrate is commonly known as clomid and it is a fertility drug used in the treatment of anovulatory disorders such as polycystic ovarian syndrome (PCOS). (Thessaloniki et al., 2008). Clomiphene citrate is a selective estrogen receptor modulator (SERM) (wheeler et al; 2019). It selectively binds estrogen receptors to reduce the negative feedback of 17-beta estradiol on the hypothalamus thus increasing the Gonadotrophin releasing hormone. (Scovell et al; 2018) LH and FSH in turn are released from the pituitary gland. These two hormones act on the ovary, and uteruses to influence the ovarian and uterine functions. CC is also largely accepted as a drug of choice for anovular cause of infertility. It has also been found it used in a short luteal cause of infertility. CC as a SERM has both estrogenic and antiestrogenic effects on estrogen-sensitive tissues (wheeler et al;2019).

There have been several animal studies on the effects of CC on ovarian functions, and several of them concluded that CC has mainly antiestrogen effects on the ovary (moon et al; 1989). Most of these works did not consider the way this drug is administered in humans. CC is given orally, from the third to the fifth day of menses. Treatment usually starts with 50 mg/day for 5 consecutive days. A maximum of 250 mg/day but above 100 mg/day is not approved by the FDA (ASRM 2006; Yilmaz et al., 2014) Whether the actions of CC will contribute to increasing or decreasing the EC parameters like estrus cycle length will further support and explain it mechanism in promoting fertility.

There are several biological rhythms in the animal system. The estrus cycle is an example of these endogenous rhythms in the body. Rats have a short estrus cycle of 4-5 days making it an important animal for study in reproduction. The changes in this cycle correspond to a lot of parenchymal changes in the ovary at puberty. Estrus cycle activities are great tools to explain events in the reproductive system of animals. In rats, the estrus cycle is divided into four phases depending on the cells present in the vaginal in an early smear. Marcondes et at.; (2002) classified the phases as proestrus, estrus, metestrus, and diestrus based on the types of cells available in the vaginal smear during the early morning hours. These phases also correspond to other activities in the reproductive system like ovarian, and uterine changes among others. proestrus corresponds with the preovulatory phase, ovulation takes place between estrus and proestrus while metestrus and diestrus represent a postovulatory phase.

There are numerous attempts at studying the estrus cyclicity. Like regularity of the cycle, interval between the phases, and number of phases of the cycle (Díaz-Duran et al; 2017) Different researchers therefore have worked with various indices like the estrus phase interval which looks at the average duration of each estrus phase, Estrus cycle length that examines the average number of days it takes to complete a cycle and diestrus index: examines the relation of diestrus phase to other phases. However, the estrous cycle ratio (ECR) is a better predictor of the cyclical phenomenon that takes place in ovarian parenchyma during puberty (Adeniyi et al; 2019)

ECR was determined by %proestrus +% estrus divided by % metestrus +% diestrus. The values relate the length of the follicular phase to the luteal phase. When the follicular phase is too short, it might affect folliculogenesis, a longer luteal phase may mean more progesterone to sustain ova release. Factors that increase the ECR will mean a prolonged follicular phase, while a decrease will promote the availability of ova for fertilization (Adeniyi et al;2019).

Studies show that CC favors ovulation in humans but prevents ovulation in rodents. This was attributed to its estrogenic and antiestrogenic properties respectively (Hawaze et al;2017). It is important to know that the subject of fertility is beyond follicular recruitment, it includes ovulation, implantation, and sustenance of the pregnancy. Each of these stages is largely affected by various factors (Holesh et al; 2023). The big question is at what point do we have CC influence fertility and at what dose in rodents? Therefore, the question of why CC is a drug of choice in anovular cause of infertility is still a subject that needs further evaluation. In this study, we want to investigate the actions of clomiphene citrate on the estrus cycle and the ova shed in the ovary of female Sprague- Dawley rats.

2.0 METHODOLOGY

Sixty female rats(100-120g) were purchased from the animal house of the College of Medicine of the University of Lagos (CMUL). The rats were housed in temperature-controlled room with alternating 12h light/12h dark cycle, and they were given food and water *ad libitum*. The rats were allowed to acclimatized for two weeks before treatment was commenced (25 ± 1 °C). The use and the animals in the study complied with the CMUL Ethical committee guidelines number CMUL/ACUREC/09/22/1088. After the period of acclimatization, estrus cycle was checked and rats with 4 to 5 days estrus cycle were selected for the study and randomly divided into five groups (n=10). *(*Marcondes et al.,2002*)* for 6 cycles.

Experimental Design

Following grouping, the rats were given various doses of CC dissolved in distilled water. Solution was administered orally using oral cannula to go through the estrus cycle six (6) times.

Group 1: Control group was given distilled water. Group 2: 0.2mg/kg of CC. Group3: 2mg/kg of CC. Group4: 4mg/kg of CC. (drug solution for groups 2-4 were given at Diestrus). Group 5: 6mg/kg/ daily. (elkhateeb *et al.*, 2017).

ECR technique: Daily vaginal smears were taken between 8am and 9am using plastic pippete with normal saline. Various estrus phases were evaluated *(*Marcondes et al.,2002*)* for 6 cycles.

ECR was calculated using the formular;

ECR= (Proestrus + Estrus) / (Metestrus + Diestrus) (Adeniyi et al.,2019).

The Ova Shed Technique

The oviduct of the rats was removed at autopsy at the estrus phase of the estrus cycle. The separated oviduct is placed between two glass slide and the ovum is counted under light microscope

Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM). The data was arranged using one -way ANOVA (Analysis of Variance) from Graph Pad prism 8 software followed by Bonferroni protest. The level of statistical significance was taken at $P < 0.05$. All results obtained in this study was statistically analyzed in other to draw a valid conclusion on the analysis involved.

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3.0 FINDINGS

Table 1: Effect of doses of CC on Estrus Phases.

Phases in Estrus Cycle

Phases	Group A (Control)	Group B (0.2 Mg/Kg)	Group C (2 Mg/Kg)	Group D (4 Mg/Kg)	Group E (6 Mg/Kg)
Proestrus	8.0 ± 0.25	2.6 ± 0.41*	3.2 ± 0.45*	2.3 ± 0.49*	3.2 ± 0.45*
Estrous	8.6 ± 0.42	2.5 ± 0.42*	2.0 ± 0.42*	1.8 ± 0.47*	2.0 ± 0.42*
Metestrus	9.6 ± 0.21	6.7 ± 0.45*	7.6 ± 1.03*	5.8 ± 0.83*	7.6 ± 1.03*
Diestrus	8.6 ± 0.21	8.1 ± 0.66NS	7.6 ± 0.62*	6.8 ± 1.12*	7.6 ± 0.62*

Values represent Mean ± SEM. **Significant *p<0.05 vs. CONTROL,**

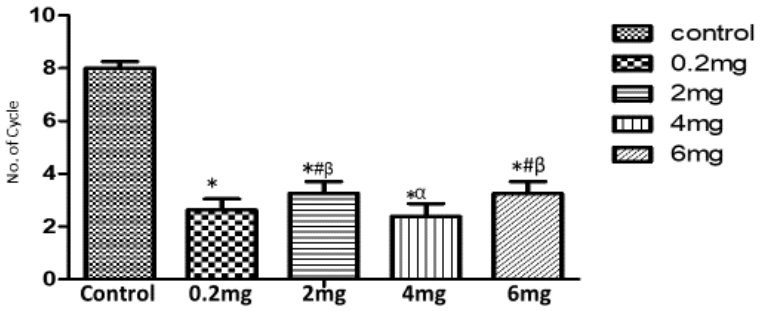

Fig 1.1 showing the number of proestrus phase of the estrus cycle at different doses of CC administration.

Values are expressed in mean ±SEM

* = P < 0.05 when compared with control # = P < 0.05 when compared with 0.2 mg

α= P < 0.05 when compared with 2mg β = P < 0.05 when compared with 4 mg

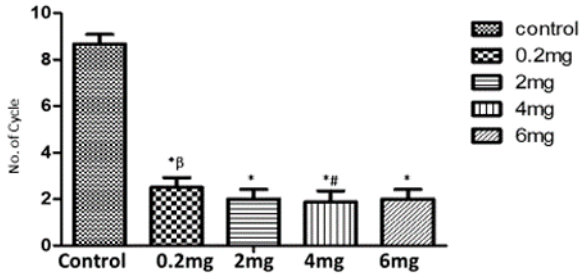

Fig 1.2.: Graph showing the number of estrus phase of the estrous cycle at different doses of CC administration.

Values are expressed in mean ±SEM

* = P < 0.05 when compared with control # = P < 0.05 when compared with 4 mg

B = P< 0.05 when compared with 0.2 mg

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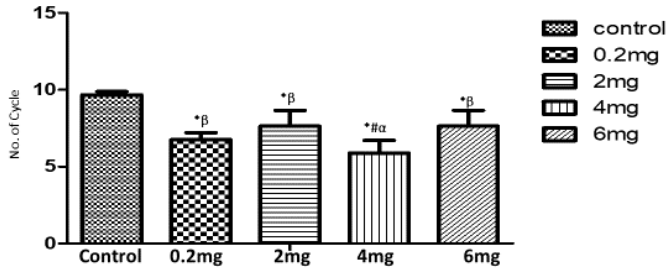

Fig 1.3.: showing metestrus phase of the estrous cycle at different doses of CC administration.

Values are expressed in mean ±SEM

* = P < 0.05 when compared with control

β = P < 0.05 when compared with 4 mg

= P < 0.05 when compared with 0.2mg

α = P < 0.05 when compared with 2 mg

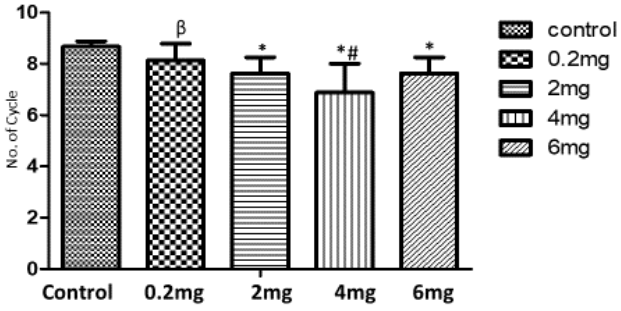

Fig 1.4: showing diestrus phase of the estrous cycle at different doses of CC administration.

Values are expressed in mean ±SEM

• = P < 0.05 when compared with control

β = P < 0.05 when compared with 4 mg

= P < 0.05 when compared with control

Table 2: Effect of Cc on %of E+P, %of M+D, Ecr.

Estrous Cycle Ratio	Proportion of (E + P) %	Proportion of (M+D) %	ECR
Group A - Control	**47.60**	**52.40**	**0.9 ± 0.03**
Group B - 0.2 mg/kg	**25.60**	**74.40**	**0.3 ± 0.04***
Group C – 2 mg/kg	**25.61**	**74.40**	**0.3 ± 0.6***
Group D – 4 mg/kg	**24.81**	**75.20**	**0.3 ± 0.08***
Group E – 6 mg/daily	**25.60**	**74.40**	**0.3 ± 0.06***

Significant *p<0.05 vs. CONTROL,

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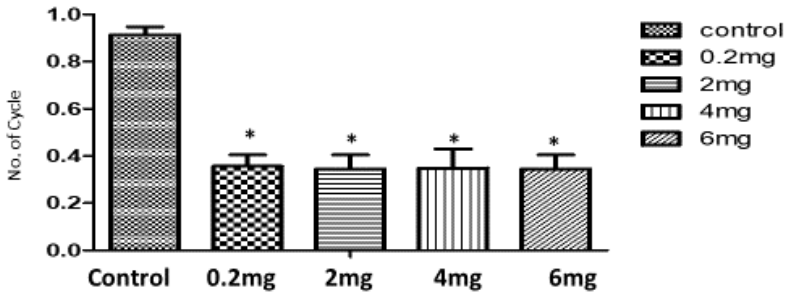

Fig 1.5.: showing ECR of the estrous cycle at different doses of CC administration.

Values are expressed in mean ±SEM

*** = P < 0.05 when compared with control**

Table: 3 Effects of CC on Animal that Shed Ova and the Number of Ova Shed

Ova Shed Count	Proportion of rats with ova shed.	Number of ova shed per group
Group A (Control)	5/5	9.7± 0.3
Group B (0.2 mg/kg)	5/5	5.0 ± 1.2*
Group C (2.0 mg/kg)	5/5	5.0 ± 0*
Group D (4.0 mg/kg)	5/5	6.7 ± 1.3*
Group E (6.0 mg/kg)	5/5	5.0 ± 0*

Significant *p<0.05 vs. CONTROL.

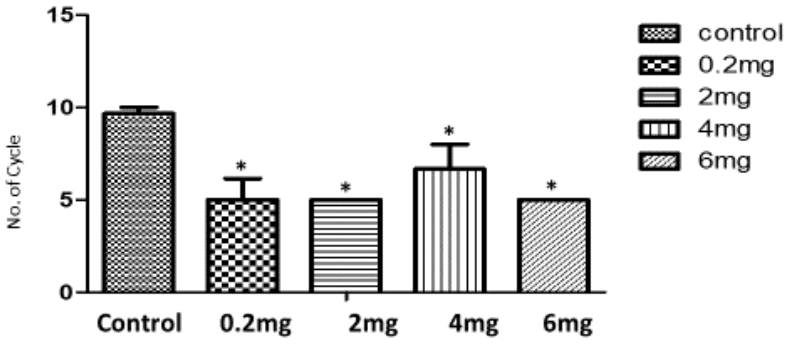

Fig 1.6 showing number of ova shed at the estrus phase of estrous cycle at different doses of CC administration.

Values are expressed in mean ±SEM

*** = P < 0.05 when compared with control**

Discussion

EC in rats is 4-5 days (Marcondes et al., 2002). Rats have normally over 90 percent DNA resemblance with humans (Aitman et al.,2008). This makes this animal an ideal for human studies. This study shows that CC reduces EC by a decrease in the number of proestrus, estrus metestrus, and diestrus compared to the control during the period under study. This is supported by a study done in humans by Peter J Bartzen; in 1968, where he concluded that CC can cause a reduction in menstrual cycle length. This would likely improve fertility by increasing the

number of cycles a patient will experience in a year assuming ovulation takes place at every cycle, more so that fertility is defined in terms of time and not cycles.

This study also indicates an obvious reduction in the ECR of the treated animals compared with the control. This is noticed when the ratio of the preovulatory phase of the menstrual cycle, represented by the proestrus and estrus phases, and the postovulatory phase represented by the metestrus and diestrus decreased significantly. Consequently, it reduces the follicular phase but increases the luteal phase. Adeniyi et al; 2019 stated that factors that will decrease ECR will likely promote ovulation.

It was further explained that a decrease in ECR will favor ovulation by prolonging the luteal phase over the follicular phase therefore making more time and or availability of the released egg for fertilization. There is a drop in ECR as noted in this study, but since these indices relate to estrus phases, its action will contribute to fertilization and not ovulation. This is largely supported by a human report by Ding et al, (2016) who stated that CC causes a prolonged luteal phase. The report further stated that the prolonged effect of CC on the luteal phase explains why this drug is used for egg collection during artificial reproduction. CC can therefore contribute to fertilization by increasing the number of cycles per year and making the egg more available for fertilization by prolonging the luteal phase than the follicular phase of the estrus cycle

while comparing CC use with ova shed. This study shows that all the rats produced ova across the groups, however, there is a significant drop in the number of ova shed in the treated animals when compared with the control. It therefore means that even with prolonged use of CC, there is still evidence of ovulation as eggs are released at high daily doses of the use of this drug. Meaning that an increase in the dose of CC will not likely stop ovulation.

In contrast to this, the number of eggs released in this work decreased across the treated group as against the control. This agrees with several works before now that links CC use to a decrease in the number of eggs released like the one by Hoitkamp et al; 1960. Most of these studies even stated that there is a difference in the response of other primates to CC regarding ovulation. Where CC produces a decrease in the oocyte released.

This work makes a distinction between the eggs released and the number of eggs released. As the egg released is not affected by CC use, the number of eggs released is greatly decreased by it. This decline in eggs released from CC use is largely supported by many authors who used animals like rats. For instance, Hawaze et al in 2017 [17] reported a reduction in ovulation since the number of antral follicles decreased with CC use. This position was backed by the hypothesis that the outcome of CC on ovulation is different in humans and other mammals. It was further believed that the reduction in the number of antral follicles by CC in rats is a result of its antiestrogenic properties. Although this position was not substantiated. It is important to know that while our work stands out when compared with another animal model CC is used in this work to mimic human use. Other animal models gave this medication daily and at higher doses, which could largely be responsible for the variation in results documented

This study therefore hypothesizes that the mechanism of egg release is different from the mechanism of the number of eggs released. Further studies may be needed to clarify what determines the number of eggs to be released during ovulation.

It is very important to be clear on the subject of fertility and CC use. CC according to this work, contributes largely to fertility by increasing the number of cycles per time assuming ovulation takes place per cycle. In addition to this, it also prolongs the luteal phase, hence making egg to be available after ovulation proper. Therefore, CC is a pro-fertility drug but may

be challenged as a pro-ovulation drug. The ovulation mechanism on the other hand has been linked to luteinizing hormone surge and dominant follicular theory among others.

4.0 CONCLUSION AND RECOMMENDATIONS

Conclusion

The use of this drug promotes luteniogenesis therefore prolonging the availability of eggs for fertilization. However, the reduction in the quantity of ova shed in this study further suggests that CC may not likely cause ovulation but contributes to the overall outcome of fertility. Couples on CC for infertility management should take advantage of the luteal phase to copulate more.

Recommendations

Further studies should be done to investigate why CC is causing a drop in the number of eggs released at ovulation. it is also important to investigate the action of CC on the whole process of female fertility, such as follicular recruitment, follicular growth, implantation, and sustenance of pregnancy to the age of viability.

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