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Effect of Acetaminophen on the Estrous Cycle and Reproductive Hormones of Female Mice

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ABSTRACT

In responding to the research question, what is the effect of acetaminophen on reproductive hormones and estrous cycles of mice? A study was undertaken to establish the potential impact of acetaminophen on the pituitary-gonadal axis. Reproductive hormones are critical drivers of regular estrous cycles in mammals. The hormones are required to achieve and maintain conception and the reproductive potential among domestic animal species. Up or down-regulation of any reproductive hormones interferes with the estrous cycle and may result in temporary or permanent infertility. Acetaminophen elicits analgesia through inhibition of prostaglandin synthesis and activation of the endocannabinoid system. This study aimed to investigate the effect of acetaminophen on the estrous cycle and reproductive hormone levels. The study utilized 6-8 weeks old female mice divided into control and treatment groups with five mice each. The control and treatment group received normal saline and 200mg/kg acetaminophen via oral gavage for 20 days, respectively. There was no difference in the length and number of cycles observed, but there was a significant reduction in the frequency of proestrus in the treatment group. There was a delay in producing estradiol, Luteinizing hormone and follicle-stimulating hormone in the treatment group. From this study, acetaminophen negatively affected the estrous cycle and hormone production in the treated mice. The observed disruption in hormone patterns could be a potential cause of infertility for both humans and animals that use acetaminophen.

Key words: Acetaminophen, Estrous cycle, Female hormones, Mice.

INTRODUCTION

Acetaminophen is readily available over the counter analgesic and anti-pyretic. Despite inhibiting cyclooxygenase (COX) production, acetaminophen is not considered as a typical non-steroidal anti-inflammatory drug (NSAID) due to its weak inflammatory properties. Due to this reason, there was limited research studies focused on its potential interference with reproduction.

The estrous cycle is a recurrent pattern of ovarian activity that enables a female animal to go from a period of reproductive receptiveness to non-receptivity with the ultimate goal of getting pregnant after mating (Crowe 2022). An interference with folliculogenesis will result in either delayed or anovulation. Ovulation is a highly regulated flow of events and can be classified as inflammatory (Duffy et al. 2019). Prostaglandins (PGs) play a role in regulatory of the estrous cycle. Inhibition of prostaglandin synthesis may be associated with reproductive failures. Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to affect the female reproductive cycle causing reversible infertility (Stone et al. 2002).

Acetaminophen competitively inhibits prostaglandin synthesis. Acetaminophen competes with arachidonic acid for the Cyclo-oxygenase (COX) enzyme (Botting 2000). Oxidation of COX is necessary to exert its enzymatic activity (Sharma and Mehta 2014). Acetaminophen acts at the peroxidase (POX) site as a reducing co-substrate, indirectly interfering with this (Sharma and Mehta 2014). Acetaminophen stimulates prostaglandin synthesis at low concentrations, and the reverse is true at high levels (Botting 2000). In this study, we postulate that acetaminophen will inhibit COX resulting in reduced prostaglandin production. This reduction in prostaglandin production will affect reproductive events such as ovulation or lysis of the corpus luteum resulting in delayed/ anovulation or a prolonged life span of the corpus luteum and consequently disrupting the estrous cycle.

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After parenteral administration, acetaminophen also undergoes deacetylation to p-aminophenol in the liver and central nervous system (CNS) (Högestätt et al. 2005). In the CNS, P-aminophenol is conjugated with arachidonic acid by the fatty acid amide hydrolase (FAAH) to produce N-arachidonoylphenolamine (AM404). AM404 is an anandamide membrane transporter blocker. It inhibits the reuptake of anandamide, creating a high concentration of anandamide within the extracellular space. Low anandamide concentration during the first steps of folliculogenesis is required for proper oocyte maturation (Cui et al. 2017). Acetaminophen, through its conversion to AM404, may lead to an increase in anandamide levels. The resulting high levels of anandamide interfere with the folliculogenesis and hence the estrous cycle.

In this study, we analyzed the effect of acetaminophen on the estrous cycle and hormone production in female mice.

MATERIALS AND METHODS

All experimental procedures and ethical considerations on the use of animals were carried out as guided by the Kenyan animal welfare guidelines and approvals obtained from the Faculty of Veterinary Medicine University of Nairobi Biosafety, Animal Use, and Ethics committee – (FVM BAUEC/2019/187). Female, Swiss white mice, aged between 8-10 weeks old and weighing between 20-35g were used for the study. These animals were purchased from the Kenya Medical Research Institute (KEMRI) and kept in cages within the laboratory. These mice were housed in groups of five and maintained under conditions of 12-hour photoperiod and at room temperature. And accessed *ad libitum* commercially obtained diet of mice pellets (Unga Limited®) and water. All the mice were subjected to an initial acclimatization period of two weeks before the start of the experiments.

The effect of acetaminophen on the estrous cycle was evaluated using ten normocyclic female mice divided into two groups (treatment and control) of 5 mice in each. The treatment group received 200mg/kg acetaminophen (Hawk et al. 2005) daily through intra-abdominal gavage, while in control group mice received 0.5mL normal saline for 20 days to observe any changes in cyclicity for 4-5 cycles. Vaginal smears were done daily through gentle lavage using phosphate-buffered saline, as Cora et al. (2015) described. The estrous cycle was staged by examining the morphology and proportion of both the leukocytes and the epithelial cells under a X40 objective light microscope as previously described (Cora et al. 2015). The length of each stage and the entire cycle was recorded. These experiments were repeated three times.

Laser Raman spectroscopy was used to determine the levels of female reproductive hormones in this study. The hormones measured were Follicle-stimulating hormone (FSH), Luteinizing hormone (LH), estradiol and progesterone. Standard female reproductive hormones were mixed with blood from male mice for identification, calibration, and quantification on Raman spectroscopy. About 5 µL of standard female reproductive hormone was measured and smeared on a silver-coated glass slide. The standard hormone concentrations used for calibration were 5mIU/mL for FSH and LH, 20pg/mL for estradiol and 0.2ng/mL for progesterone. Blood was collected from the tail vein daily from the studied mice and applied on a silver-coated slide for Raman spectrometry hormone assay.

The Artificial Neural Networks (ANN) predictive models were made using identified spectral bands from feature selection in R software. The ANN models were then used to determine the hormone levels in blood from mice in both groups. The predicted concentrations were then recorded and analyzed by the stage of the estrous cycle.

Collected data were analyzed using SPSS version 12.0 for windows (SPSS, Chicago, Illinois, USA). T-Test examined differences of means between two groups, $P \leq 0.05$ was considered as statistically significant.

Table 1: Average number of cycles and length of estrous cycle (mean+SE) observed over 20 days

Parameter	Run	Control (n=5)	Treatment (n=5)
No of cycles	1	3.7±0.274	3.0±0.274
	2	3.2±0.245	3.4±0.245
	3	3.75±0.274	3.2±0.245
	Average	3.54±0.144	3.21±0.155
Length of cycles	1	4.92±0.32	5.75±0.32
	2	5.86±0.286	5.4±0.286
	3	5.25±0.32	5.47±0.286
	Average	5.38±0.172	5.52±0.187

Table 2: Appearance frequency of estrus cycle stages (mean+SE) during the 20-day acetaminophen administration

Stage of Estrous	Control (n=13)	Treatment (n=14)
Proestrus	4.77±0.323	3.79±0.281*
Estrus	5.31±0.365	6.21±0.408
Metestrus	4.38±0.266	4.36±0.551
Diestrus	5.23±0.579	5.36±0.599

*Significant difference at the same row ($P<0.05$).

RESULTS AND DISCUSSION

There was no difference in the length and number of estrous cycles observed over the 20 days in all the runs (Table 1). However, there was a significant reduction in the frequency of proestrus stage in the treatment group as compared to the control group (Table 2). There was no difference between the treatment and control group in all the other estrous cycle stages.

There was no difference observed in the levels of estradiol, FSH and LH between the control and treatment group when analyzed by group and stage of the estrous cycle (Table 3). There was a difference in the pattern of production for these three hormones in the treatment group with peak production during metestrus as opposed to estrus (Fig. 1). The levels and pattern of production of progesterone did not differ between the two groups. However, there were significantly higher levels of progesterone during metestrus in both groups $P \leq 0.034$.

This study mainly utilized mice due to their short estrous cycle duration and that mice have the highest level of uterine anandamide (Paria et al. 1996). The mice in this study had regular estrous cycles during the 20 days of experiment. The estrous cycle in mice averages 4–5 days (Cora et al. 2015). There was no difference in the length and number of estrous cycles in the control and treatment groups. However, the length of the estrous cycle was longer than the documented average; this may be due to housing. As, it was observed that mice housed alone tend to have regular and consistent 4/5 day cycles compared to mice

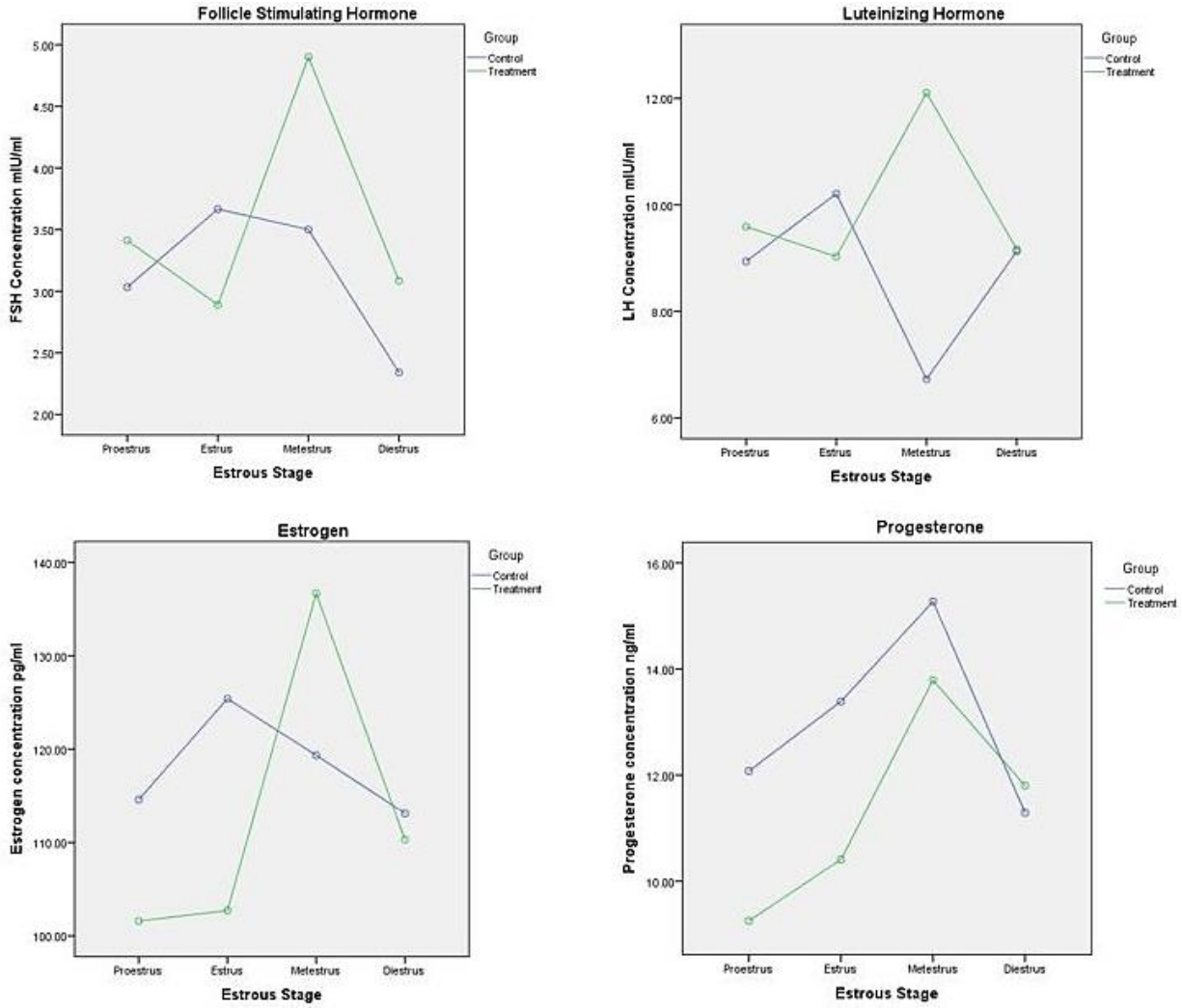

Fig. 1: Figure showing the production pattern of female reproductive hormones during different stages of the estrous cycle.

Table 3: Raman spectral data table showing the average levels of female reproductive hormones

Estrous cycle	FSH (mIU/mL)		LH (mIU/mL)		Estradiol (pg/mL)		Progesterone (ng/mL)	
	Control (±0.30)	Treated (±0.46)	Control (±2.77)	Treated (±8.18)	Control (±0.73)	Treated (±0.72)	Control (±0.87)	Treated (±0.98)
Proestrus	3.033	3.413	114.59	101.59	8.939	9.591	12.08	9.252
Estrus	3.666	2.890	125.39	102.73	10.21	9.029	13.39	10.41
Metestrus	3.500	4.900	119.34	136.67	6.728	12.10	15.27	13.79
Diestrus	2.341	3.085	113.13	110.31	9.130	9.166	11.29	11.80

housed in groups as in this study (Cora et al. 2015). As observed in this study, the estrous cycle is divided into four phases: proestrus, estrus, metestrus, and diestrus. The use of wet mounts for direct microscopy to evaluate estrous cycle stages in this study agrees with other studies (Caligioni 2009; Cora et al. 2015). This study was the first to report the effect of acetaminophen on the estrous cycle of mice.

The frequency of proestrus was reduced significantly in the treatment group as compared to the control. Each stage lasts about 6-72 hours, depending on the stage and individual mouse. Therefore, some short stages like proestrus may be 'missed' mainly if sampling is done very early or late in the day (Cora et al. 2015). However, this is unlikely given that sampling was done at the same time for both groups. A more likely explanation could be that follicles undergo premature luteinization bringing the proestrus phase to an early end. Proestrus in mice lasts about 12-24 hours. Luteinized unruptured follicles (LUFs) occur when the antral follicle fails to ovulate (Bashir et al. 2016). Several prostaglandin inhibitors cause luteinization of follicles in both animals and women (Bashir et al. 2018). Prostaglandins produce proteolytic enzymes, which help in follicular rupture (Kim et al. 2014). Therefore, inhibition of the COX enzyme by acetaminophen might lead to persistent follicles and ovulatory failure, as discussed by Bashir et al. (2018).

Like NSAIDs in other studies, acetaminophen did not affect the levels of FSH, LH, and Estradiol (Al-Atraki et al. 2012; Martini et al. 2008). Gonadotropin-releasing hormone from the hypothalamus influences the anterior pituitary to release FSH and LH (Christensen et al. 2012). At the same time, estrogen is produced by developing follicles. Typically, estradiol levels rise during proestrus,

continue to increase during estrus and reach maximum levels just before ovulation, then decline during metestrus before increasing again during diestrus (Caligioni 2009). A rapid increase in plasma estradiol concentration during proestrus generates a positive feedback mechanism that triggers a surge in GnRH secretion (Miller and Takahashi 2014). In turn, GnRH stimulates the production of LH from the anterior pituitary gland, which then triggers ovulation (Miller and Takahashi 2014). In this study, the FSH, LH, and estrogen levels were highest during metestrus in the treatment group, which is a deviation from the normal. The observed changes indicate that this cascade was delayed, and peak hormone levels were attained during metestrus instead of estrus. This delay might have led to late ovulation. Delayed ovulation is one of the adverse effects of acetaminophen in mice leading to the reduced number of implantation sites (Ndeke et al. 2021). These results have shown that Raman spectroscopy and chemometrics can measure hormone levels in the blood.

Conclusion

From this study, acetaminophen negatively affected the estrous cycle and hormone production in the treated mice. The observed disruption in hormone patterns could be a potential cause of infertility to both humans and animals that use acetaminophen. The results presented here apply to both human and veterinary medicine. However, further research is needed to understand the exact mechanism of action of acetaminophen clearly.

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Author's Contribution

Dr Anne Ndeke is a PhD student under the guidance of Prof. Mutembei and Dr's Kaingu and Muthee. Dr. Ndeke performed all the experiments and the results were analyzed and interpreted under the guidance of the three supervisors. Dr Birech is in the physics department and was involved in the processing, analysis and interpretation of Raman spectra data.

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