

Immunohistochemical Study on Localized Oestrogen Receptors Following Subacute Oral Administration of 3-Methylmorphine (Codeine) in Ovaries of Albino Wistar Rats

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Abstract

The immunohistochemistry of localized oestrogen receptors following subacute oral administration of dihydrocodeine in ovaries of albino Wistar rats was studied. The specific objectives were; to determine the distribution of oestrogen receptors in the ovaries using immunohistochemical expression method and to evaluate oestrogen staining intensity using Image J statistical software. The twenty 20 rats were divided into 4 groups of five rat each. The control (group A) rats received distilled water per body weight while group B-D received 30, 60 and 90 mg/kg dihydrocodeine orally for 28 days. Animals were anesthetized using ketamine injection and abdominopelvic incision was made. The ovaries were harvested and processed using histochemistry. Photomicrographs were obtained using light microscope and arm scope. Oestrogen staining intensity was analysed using analysis of variance (ANOVA), followed by Post Hoc test using Image J statistical software. The results obtained show that the ovarian follicles had high staining intensity with monoclonal oestrogen receptor antibodies in group I, while group II showed moderate-high staining intensity. Group III showed low intensity of staining of oestrogen in the ovaries of the rats, while group IV showed low staining intensity of oestrogen in the ovarian follicle of albino rats. Oestrogen receptor staining intensity decreased in the ovaries of the treatment groups, which was significant at $p < 0.05$. This study concludes that Dihydrocodeine caused significant decrease ($p < 0.013$) in localized estrogen receptor in the ovaries of albino rats.

Keywords: Dihydrocodeine, 3- Methylmorphine, Oestrogen Receptors, Ovaries, Albino rats.

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INTRODUCTION

Opioids are used for the management of pain; they are believed to be more effective than non-steroidal anti-inflammatory drugs (Slater *et al.*, 2010; Hersh *et al.*, 2020). Dihydrocodeine (DHC) is synthesized from opioids which was developed in 1900s and has a pharmacokinetics similar to that of codeine (Webb *et al.*, 2001; Leppert, 2010). DHC is a semi-synthetic analogue of codeine formed by hydrogenation of the double tie in the main chain of the codeine molecules (Zhu *et al.*, 2022). When compared to codeine, dihydrocodeine contains a single bond instead of the double bond between carbon 7 and 8 (Wojciech and Jaroslaw, 2016).

Codeine may be placed on the second step on WHO analgesic ladder as a 'weak' opioid (Wojciech and Jaroslaw, 2016), and is being deployed as analgesic, antitussive, antidiarrheal, anti-cough and in opioid addiction (Rock *et al.*, 2022). DHC administered orally have similar analgesic potency to codeine but twice as potent as tramadol (Leppert and Majkowicz, 2010). Common side effects of dihydrocodeine include; dry mouth, sleepiness, stomach pain, nausea and vomiting (Leppert and Majkowicz, 2010), others include; fatigue, loss of coordination, sedation, dissociation, altered level of consciousness (Williams *et al.*, 2002). Most opioids caused decreased libido, difficulty in sexual activity which may be related to its effects on the release of gonadotropins (Wiffen *et al.*, 2019). In elderly patients it is associated with confusion, hallucinations, convulsion, headaches and vertigo (Wojciech and Jaroslaw, 2016).

The Nigeria government has banned the production, sale and use of codeine and codeine containing products following a report by the British Broadcasting Corporation (BBC) African Eye who carried out an undercover investigation and discovered that 3 million bottles of codeine cough syrup were consumed in just two states in northern Nigeria (BBC, 2018). The report moved the Nigerian government through its legislatures (Senate) to ban the drug in 2018. Despite the ban, codeine is still abused mostly by young people in their reproductive age. Both male and females abused codeine (Akande-Sholabi *et al.*, 2019). Other abusers of codeine include lesbians, gays, bisexuals and those that have used new psychoactive drugs (Agnich *et al.*, 2013). Thugs, criminals such as *terrorists* and those that do hard labour also abuse codeine (Akande-Sholabi *et al.*, 2019). Dihydrocodeine and other products containing codeine are still available as prescription only drug and for research purposes in Nigeria. Despite its ban in 2018, the drug is still abused. Codeine use and abuse has been associated to an increase in mental illness, liver damage, renal failure and even death in Nigeria (NOI, 2019). Nigeria as a country has lost resources in the fight, control, treatment of illnesses related to codeine abuse (BBC, 2018).

Codeine has been implicated in loss of libido and sexual difficulty. Despite the above-mentioned side effects of codeine, the negative effect of codeine on the female ovary has been poorly examined. Hence, this study aimed at determining the effect of dihydrocodeine on immunohistochemically localised oestrogen receptors following its subacute oral administration in the ovaries of albino Wistar rats.

METHOD

Materials Employed

Dihydrocodeine tablets (Actavis UK) 30 mg, distilled water, animal cages and drinkers, animal feed (vital feed, Grand cereal Jos), syringe and intubation tubes, electronic weighing balance (BOSCH India), specimen bottles, and cover-slips, microscope (Olympus, Germany), rotary microtome (Matler, Germany), reagents of different kinds (Neutral buffered formalin,

DPX mountant, xylene, paraffin wax, hand gloves, monoclonal mouse anti-human estrogen receptor; Ready to use (Dako, Glostrup, Denmark), Super frost plus slide, hydrogen peroxide, methanol, normal horse serum, microwave oven, citrate buffer, peroxidase enzyme, streptavidin ABC kits (DAKO), 3,3 diaminobenzidine substrate, and hematoxylin.

Experimental Animal Model Employed

Twenty (20) female albino Wistar rats were used for this study, weighing between 120 to 140 grams were obtained from the laboratory animal holdings, Department of Anatomy, Bayero University Kano, Nigeria. Experimental rats were kept in the animal house of the department of Anatomy and maintained on standard pellet diet and water *ad libitum*. The rats were housed 5 per cage. The rats were kept in well ventilated plastic cages covered with wire mesh and housing with average humidity, with a temperature range of between 27 - 30 ± 2°C. The lighting condition consists of natural day light: darkness rhythm.

Experimental Design

The twenty (20) rats were divided into four (4) groups (I, II, III and IV). Each group comprised 5 rats each, which were weighed and grouped randomly. Group I served as the control group which were not administered codeine for the period that the experiment lasted. Group II rats were administered 30mg/kg dihydrocodeine by orogastric intubation daily for 28 days. Group III rats received 60 mg/kg dihydrocodeine. Group IV rats were administered with 90 mg/kg of dihydrocodeine orally and daily.

Technique Employed for Histochemistry of Estrogen Receptor

Tissues were processed for paraffin section into tissue blocks and cut between 3-5 microns and mounted on super frost plus slides. Sections were then dewaxed in xylene and hydrated in descending series of alcohol. Sections were blocked with 3% Hydrogen peroxide (H₂O₂) in methanol for 10 minutes. Sections were washed for 5 minutes and then blocked for non-specific binding sites with normal horse serum and pressure cooked for 4 minutes at 100 °C in an oven and immersed in citrate buffer at a pH 6. It was allowed to cool down for 30 minutes and washed in running tap water for five minutes. Sections were incubated with streptavidin ABC antibody for 30 minutes at room temperature. Sections were also incubated with 5 ug/ml estrogen receptor antibody at 4°C overnight. 3,3 diaminobenzidine (DAB) substrate was apply for 10 minutes and counterstained with hematoxylin, dehydrated in alcohol and cover slipped (Katoh *et al.*, 1997). Micrographs were taken using the light microscope at X 40 and X 100 magnifications.

Statistical Analysis

Estrogen receptor staining intensity was analyzed using Image J statistical software (Crowe and Yue, 2019). One-Way Analysis of Variance (ANOVA) was used to determine the differences in the measured parameters across the study groups. A Bonferroni post-hoc test was carried out to identify the comparative group responsible for the significant difference identified in the ANOVA test.

Ethical Consideration

Ethical clearance was obtained from the animal ethical committee, Bayero University Kano (BUK/CHS/HREC/125). Animal handling was based on the guideline of the Institute of Animal Care and use committee (IACUC) guidelines (Anderson, 2002) and the Bayero University ethics committee.

RESULTS

Effects of dihydrocodeine on the histochemistry of the ovary

The study revealed a significantly ($p < 0.05$) increased percentage of immune-positive estrogen receptors in the control rats compared with the experimental rats administered with dihydrocodeine. The significant ($p < 0.05$) decrease in the percentage of immune-positive estrogen receptor expression caused by dihydrocodeine was dose-dependent. Rats in group III had significantly higher immune-positive estrogen receptors compared with rats in groups II and IV (Figures 1, 2 and 3).

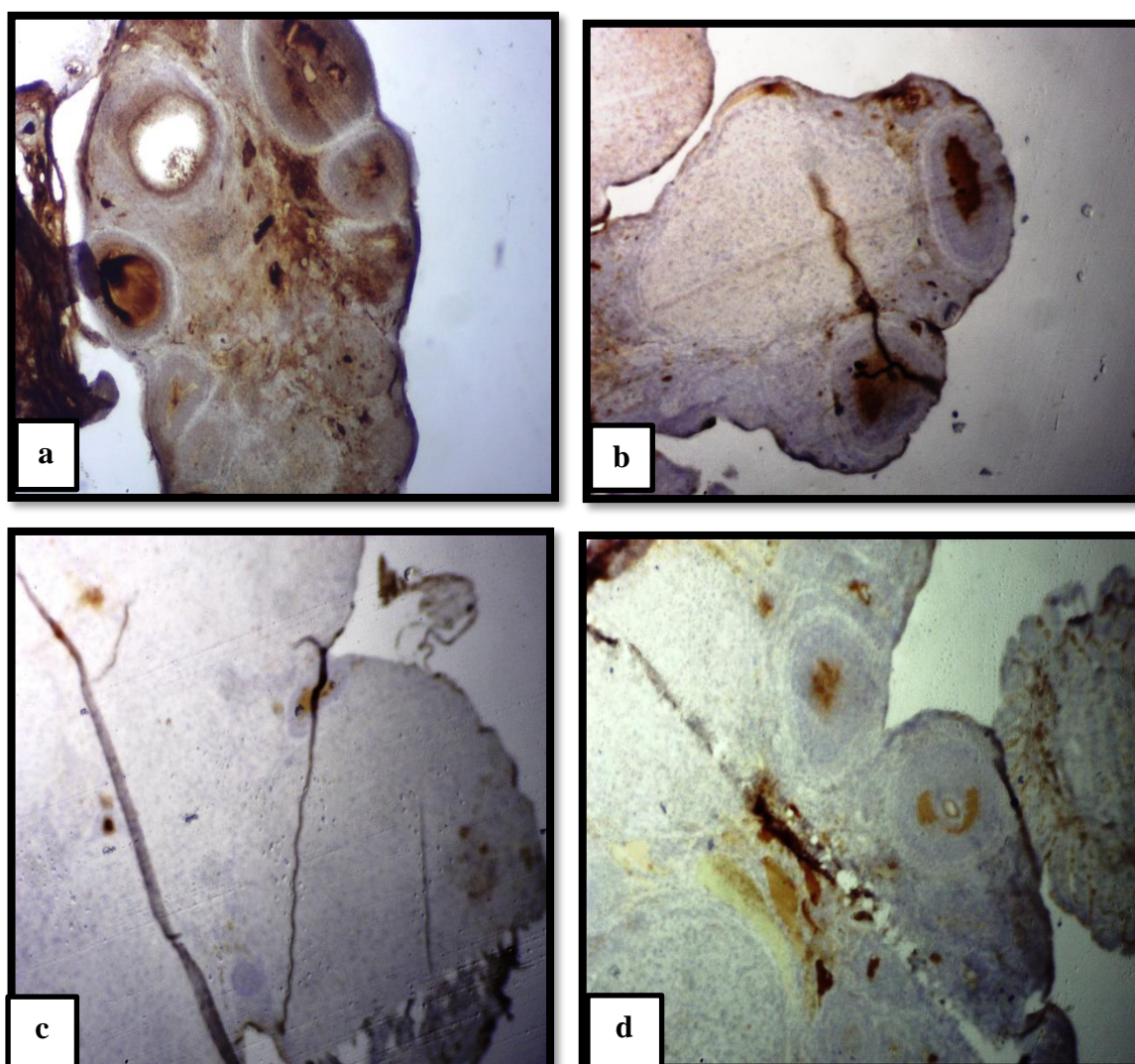


Fig 1: Effect of Codeine on the Histology of Rat Ovary Photomicrograph (Group A, B, C and D): Immunochemically Stained estrogen receptor (ER) x40. **a.** Group A (Control) Showing High Intensity of Localized Estrogen of Ovarian Follicles. **b.** Group B Showing High Coloration of Localized Estrogen Receptor in the Ovary. **c.** Rat Ovary of Group C Showing Moderate Coloration of Localized Estrogen Receptor in the Ovary. **d.** Group D Showing Moderate Coloration of Localized Estrogen Receptor in the Ovary

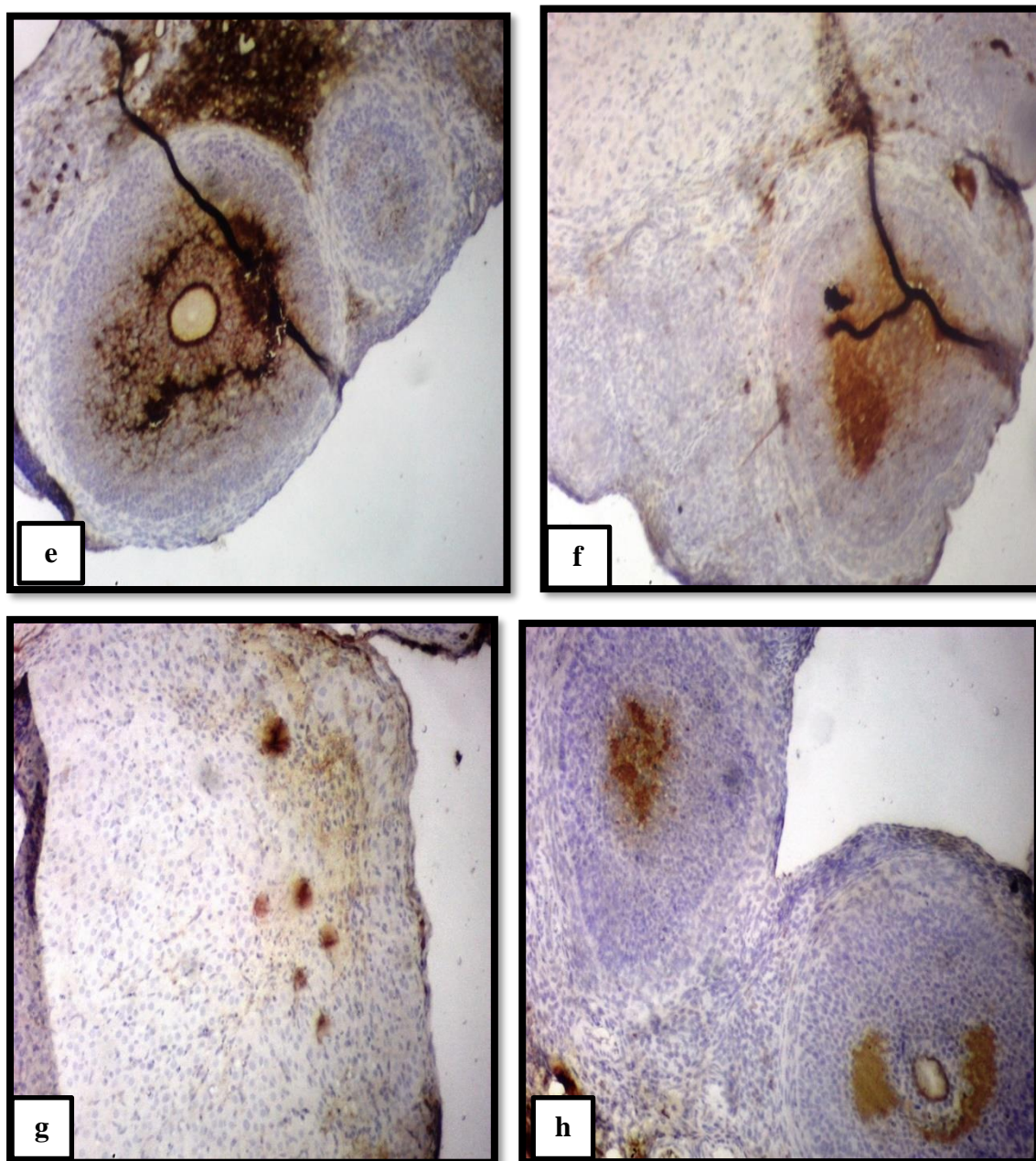


Fig 2: Effect of Codeine on the Histology of Rat Ovary Photomicrograph (Group A, B, C and D): Immunochemically Stained estrogen receptor (ER) x100. **e.** Group A Showing High Coloration of Localized Estrogen Receptor. **f.** Group B Showing Moderate Coloration of Localized Estrogen Receptor in the Ovary. **g.** Group C Showing Low Coloration of Localized Estrogen Receptor. **h.** Group D Showing Low-Moderate Coloration of Localized Estrogen Receptor in the Ovary of Albino Rats.

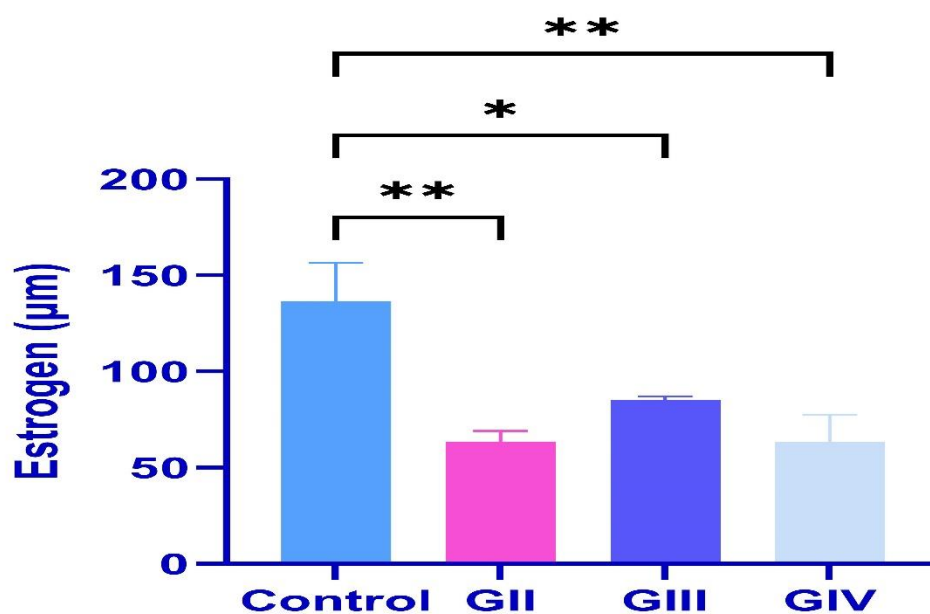


Fig 3: Effect of Codeine on the Staining Intensity of Estrogen Receptor Antibody in the Ovaries of Albino Wistar Rats. N=5, G= Group Mean \pm SEM; one-way ANOVA, Tukey *post-hoc*; $p < 0.05$ Across the Groups.

DISCUSSION

Despite the fact that DHC is used to treat chronic pain, little is known about its pharmacokinetics, particularly when the amount of DHC is increased over time. Our findings show that the pharmacokinetics of DHC and its active metabolite had a negative effect on estrogen receptor expression in the ovary of female Wistar rats in the dose range studied (30–90 mg/kg). There was a significant (Figure 3) decrease in intensity of immune-positive estrogen receptors in all the rats treated with DHC, when compared with rats in the control group. The results obtained were probably due to the feedback mechanism of the gonadotropic hormones or the direct effect of codeine on the cells producing estrogen. Earlier studies by Ajayi and Akhigbe (2020) reported that codeine induced down regulation of estrogen signaling which caused gonadal-toxicity in adult male Wistar rats. Similarly, DHC is a semisynthetic opioid, and prolonged opioid usage is known to produce severe GH shortage, which may impact estrogen receptor expression (Seyfried and Hester, 2012). An opioid effect on GH is corroborated by research in human lymphoblastoid IM-9 cells, which found that opioids dramatically affected GH receptor gene expression (Vuong et al., 2010; Leppert and Woron, 2016).

CONCLUSION

This study concludes that codeine caused a decrease in the stain intensity of localized estrogen and a significant decrease in the percentage of immune-positive estrogen receptor expression in the ovaries of albino Wistar rats.

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