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In-vivo Mice Pre-Implantation Embryo Development after Oral Administration Ethanolic Extract of Cogon Grass Roots (*Imperata cylindrica L)*

(PERKEMBANGAN EMBRIO PREIMPLANTASI SECARA IN VIVO SETELAH PEMBERIAN EKSTRAK ETHANOL AKAR ALANG-ALANG (IMPERATA CYLINDRICA L) PER ORAL)

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ABSTRACT

Cogon grass *(Imperata cylindrica L)* is known as a medicinal plant that is scattered almost worldwide. Despite its role that inhibits another plant's growth, cogon grass possesses several benefits in health. This research has to identify the effect of short-term gavage ethanolic extract of cogon grass roots (CGG) to *in-vivo* mice preimplantation embryo development. A total of 60 female mice were divided into control and treatment groups, dosages at 90 and 115 mg/kg of body weight of CGG, orally gavage for 20 days. The superovulation of mice was done at the end of the CGG treatment by injecting 5 IU Pregnant Mare Serum Gonadotropin (PMSG) and after 48 hours, followed by 5 IU Human Chorionic Gonadotropin (hCG) injection and directly the mice were mated. The mating rate was checked by the appearance of the vaginal plug 12 hours after hCG injection. Mice were sacrificed, the oviducts and cornua of uteri were isolated to collect the oocytes and embryonic cells by flushing the oviducts and cornua uteri with Phosphate-buffered saline (PBS). The effects of CGG as an antifertility were evaluated by measuring the number of oocytes, fertilization, and *in-vitro* embryo development rates. The results showed significantly reduced about half of the mating rate in the 115 mg/kg BW group ($p<0.05$) compared to control. However, the 90 mg/kg BW dose reduced 20% mating rate compared to control, and not significant ($p>0.05$). In all treatment groups, only half oocytes fertilized. The cleavage and blastocyst rate in 115 mg/kg BW group were significantly lower compared to the control group ($p<0.05$). In conclusion, oral gavage of cogon grass root ethanolic extract disrupts the mating process and development of *in-vivo* mice preimplantation embryo development.

Keywords: *Imperata cylindrical L;* mating rates; preimplantation development of the embryos; antifertility agent

ABSTRAK

Alang-alang *(Imperata cylindrica L)* dikenal sebagai gulma dan telah dimanfaatkan oleh masyarakat sebagai tanaman obat. Penelitian ini bertujuan untuk mengetahui efek jangka pendek pemberian ekstrak akar alang-alang terhadap perkembangan implantasi embrio. Mencit betina sebanyak 60 ekor dibagi menjadi tiga kelompok yaitu kontrol (aquadest), dosis 90 mg/kgBB, dan 115 mg/kgBB yang diberikan selama 20 hari. Superovulasi dilakukan dengan menyuntikkan 5 IU *Pregnant Mare Serum Gonadotropin (PMSG)* dilanjutkan 48 jam kemudian dengan injeksi 5 IU *Human Chorionic Gonadotropin* (*hCG).* Mencit segera dikawinkan lalu diperiksa keberadaan sumbat vagina setelah 12 jam untuk menentukan persentase keberhasilan kawin. Mencit dikorbankan nyawanya untuk isolasi oosit dan embrio yang diambil dari oviduk pada 14 jam pascainjeksi hCG untuk menghitung persentase jumlah oosit, oosit yang mengalami fertilisasi, dan 24 jam pasca hCG kornua uteri di bersihkan (*flushing*) dengan menggunakan *Phosphate-buffered saline* (PBS) untuk mendapatkan oosit dan embrio. Evaluasi dilakukan dengan melihat jumlah oosit, jumlah oosit yang terbuahi, jumlah embrio yang berkembang hingga blastosit. Pada dosis 115 mg/kgBB jumlah mencit yang melakukan perkawinan hanya setengah apabila dibandingkan dengan kelompok kontrol ($p<0.05$). Secara umum, terjadi penurunan perkembangan embrio secara *in vivo* pada kelompok perlakuan. Pada dosis 115 mg/kgBB, penurunan yang terjadi cukup signifikan dibandingkan dengan kontrol ($p<0.05$), sedangkan pada dosis 90 mg/kgBB terjadi penurunan sebesar 20% dan tidak signifikan ($p>0.05$). Sebagai simpulan, pemberian ekstrak akar alang-alang pada mencit menurunkan laju perkawinan dan perkembangan embrio pre implantasi.

Kata-kata kunci: *Imperata cylindrica L;* laju kawin, perkembangan embrio praimplantasi, agen antifertilitas

INTRODUCTION

Currently available data in 2017 indicate at least 206 million pregnancies recorded in developing regions, which 43% are unintended pregnancies, and about 84% of all unintended pregnancies in developing regions have an unmet need for effective contraception (Karra, 2016). The solution of family planning program to prevent population boom in the world by modern contraceptives have been implemented with aimed to prevent future unwanted pregnancy and as birth control by limiting the space between children (Hull and Mosley, 2009). Oral contraceptives are the most artificial contraception selected because the price is cheaper than other contraceptive methods and available in the market, but it has limited success or side effects (Agarwal and Allan, 2010). Synthetic oral contraceptives have many side effects such as nausea, dizziness, headaches, stomachaches, vomit, and severe side effects such as breast cancer, cervical cancer, thrombosis, direct impact on the brain and infertility in rare case (Shukla and Jamwal, 2017). Current safe and minimal side effect contraception are urgently needed.

Previous studies have been done to investigate herbal medicine originated from plants with natural ingredients for fertility (Agarwal and Allan, 2010). Some herbal contraceptions have been investigated, but they are less potent than the synthetics. For instance, *Azadirachta indica (Neem), Labisia pumila (Fatima grass), Mentha pulegium (pennyroyal), Plumbago rosea (leadwort), Hibiscus rosasinensis (chinese hibiscus), Trichosanthes cucumerina (snake gourd), Curcuma longa (turmeric)* exhibit anovulatory, antiestrogenic, antiimplantantation, and antifertility by modifying hormonal balance lead to disturbance normal menstrual cycle follicle growth (Bala 2014, Shweta *et al*., 2011).

In our previous study, ethanolic extract of cogon grass roots has the ability increase secondary sperm abnormal morphology (Widyastuti *et al*., 2018), decrease sperm concentration (Lubis *et al*., 2018) prolonge diestrus phase (Robianto *et al*., 2019), induced disruption on testis interstitial area and seminiferous tubule (Widyastuti *et al*., 2020) and disrupt folliculogenesis in mice (Widyastuti *et al*., 2020). However, there is a lack of information about the effect of ethanolic extract of *I. cylindrica* roots to *in-vivo* mice embryo development. This research has aimed to examine the effects of short-term gavage of ethanolic extract of cogon grass roots in mice mating rate, the number of ovulated mice oocytes and the development of in-*vivo* mice preimplantation embryo.

RESEARCH METHODS

Preparation of Plant Extract

Cogon grass roots were purchased from Solo, Central Java, Indonesia. Cogon grass roots were macerated by ethanol 95% for 72 hours, filtrated with a vacuum filter, and concentrated in a vacuum evaporator. The concentrated extract was suspended with carboxymethylcellulose (CMC) 0.5% and separated to the concentration of 90 mg/kg and 115 mg/kg of body weight as described in previous research (Widyastuti *et al*., 2020).

Experimental Design

This study was designed for four weeks, randomized allocation, placebo-controlled, parallel-group experimental trial to evaluate the effect of ethanolic extract cogon grass roots (CGG) in *in-vivo* mice preimplantation embryo development (*Mus musculus albinus*). The study protocol was approved by Ethics Review Committee Faculty of Medicine, Universitas Padjadjaran (No.1326/UN6.KEP/EC/2018)

Animals Subject

A virgin female mice DDY (Bio Farma, Bandung, Indonesia), aged eight weeks, with normal estrus cycle (average 4–6 days of proestrus, estrus, metestrus, and diestrus phases) weighing about 25-40 g were selected (Widyastuti *et al*., 2020). Mice were acclimatized for seven days before the experiment. Animals were housed in plastic cages (50 cm long x 40 cm x 15 cm high, 5 mice each cage) under the 12/12 light-dark cycle and were fed with standard food and tap water.

The 60 female mice were randomly grouped into three groups, each group consisted of 20 female mice. Mice in Group A received 0.5% carboxymethylcellulose (CMC) by gavage, whereas mice in group B and C received 90 mg/kg body weight (BW) and 115 mg/kg BW of CGG per day, respectively.

Superovulation

Animals were stimulated after 20 days of oral administration of CGG with 5 IU PMSG and after 48 hours followed by 5 IU hCG intraperitoneal (IP) injection. To evaluate the number of ovulated and fertilized oocytes, development of *in-vivo* embryo preimplantation, the stimulated female mice were mated with male mice with a ratio of 2:1 immediately after hCG injection (Luo *et al*., 2011).

Evaluation of Mating Rates

The next morning after 12 hours of hCG injection, the animals were checked for the appearance of the vaginal plug to examine the mating rate (Behringer *et al*., 2016; Pijnenborg, 2015). The following reproductive parameter was then calculated based on the previous study (Shah *et al*., 2016) with the formula: % of mating rate = [(number mated) x (total female mice in each group)$^{-1}$] x 100%.

The animals were weighed and sacrificed with anesthetic isoflurane with drap jar method before cervical dislocation (Flecknell, 2009a, Flecknell, 2009b). The female mice were sacrificed several days periodically according to the development of mice embryos during the preimplantation stage.

Evaluation of The Oocytes, Fertilization Rates, Cleavage, and Blastocyst Embryos

The oocytes and the fertilized oocytes were collected from the oviducts *(tuba fallopian)* and uterine, while the preimplantation embryos collected form oviduct for cornua uterine. The oviducts and cornua uterine were isolated and cleared from adhering tissues by flushing the oviducts with phosphate buffer saline (PBS) solution (Schatten, 2004). The cells were collected in petri-dish and observed under a stereomicroscope to determine the number of cells produced. Oocytes and fertilized oocytes were observed 14 hours post hCG injection. The cleavage embryos were collected 38-40 hours post hCG injection. Blastocyst embryos were collected 114-116 hours after hCG injection.

Statistical Analysis

All the values were expressed as mean. The degree of significance was set at $p<0.05$ relation to control and standard using Kruskal Wallis. All the analyses were carried out using GraphPad software 7th version.

RESULTS AND DISCUSSION

The developmental of mouse embryo *in vivo* illustrated in Figure 1. The administration of the CGG extract for 20 days significantly decreased the mating rate in Group C compared to group B and control ($p<0.05$). Whereas in Group B showed no significant difference compared to control (Figure 2).

The number of ovulated oocytes was significantly reduced in groups B and C

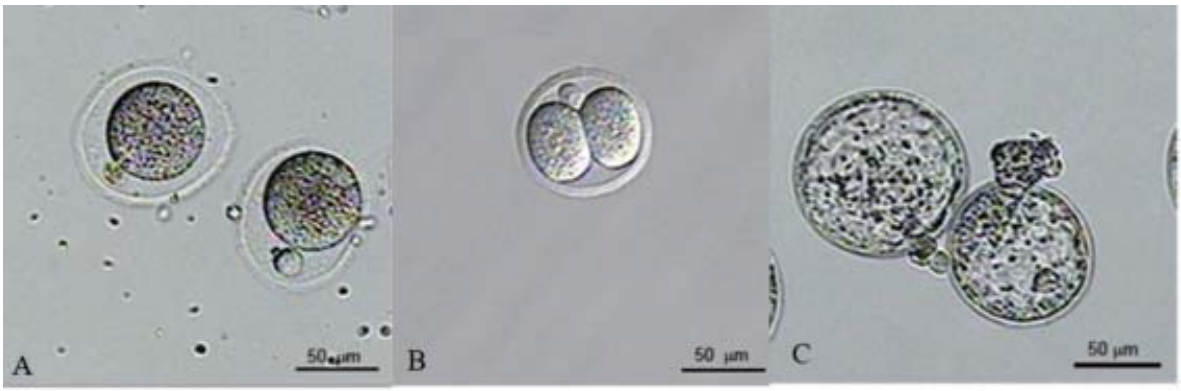

Figure 1. Developmental of mouse embryos *in vivo*. (A) mature oocytes, (B) cleavage embyros, (C) Blastocyst embryos

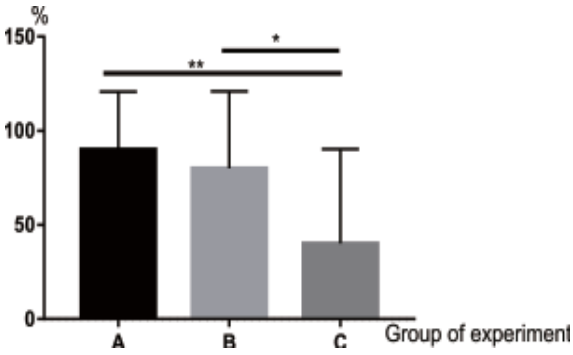

Figure 2. The effect of 20 days oral administration *Imperata cylindrica* L roots extract on mating rate of female mice. (A) control group, (B) 90 mg/kg b.w, (C) 115 mg/kg b.w.

consecutively compared to control (Figure 3a, $p<0.05$). Furthermore, the development of *in-vivo* embryos also decreased in dose depending pattern. Merely in all groups, most of the oocytes undergone fertilization (Figure 3b). Looking at the embryo development, Group B and Group C showed about a half and one-third reduction of cleavage rates, respectively compared to control (Figure 3c, $p<0.05$). Seven from nine of cleavage cells progress to blastocyst from the control group, whereas in group B and C only half of the cleavage develops into a blastocyst (Figure 3d). Overall results showed a decreasing tendency of *in-vivo* mice preimplantation embryo development in treatment groups compared to control with the highest reduction in group C.

In the present study, *I. cylindrica* L. root ethanolic reduce mice fertility effect by decreasing their mating rates, the number of oocytes produced, and *in-vivo* development preimplantation embryos. The decreased mating rate may be induced by the change of the treatment group's estrus phase duration. The previous study reported that the oral administration of *I. cylindrica* L. induced prolonged diestrus phase (Robianto *et al*., 2018). In the diestrus phase, corpus lutea secretes progesterone to prepare the endometrium for implantation and estrogen produced by granulose cells to thicken the endometrial lining. If this stage is prolonged, estrogen could not attain the maximum level which leads to Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) surge thus, no ovulation occurred. Duration of estrus phase also shortened, which implies decreased chance time for mating (Yakubu *et al*., 2010).

The results showed that the gavage of *I. cylindrica* L. decreased the number of oocytes and *in-vivo* embryo development. The disturbance may cause a decreased number of ovulated oocytes in folliculogenesis. The previous study reported that the short term gavage *I. cylindrica* L. reduces FSH serum level, increases corpus luteum number, and prolonged diestrus phase, thus impair folliculogenesis (Widyastuti *et al*., 2020).

The lower number of cleavage and blastocyst strongly correlated with the decrease of oocytes number and fertilized oocytes in a group of treatment compared to control. The decrease in embryo development might be due to antiproliferative, cytotoxic, and antiimplantation effects of flavonoids, alkaloids, and saponins. Due to *I. cylindrica* L. root ethanolic extract that was given for several days to female mice, they might not be able to eliminate the extract's active compounds in blood vessels. Therefore, it can affect the development of the embryos by its

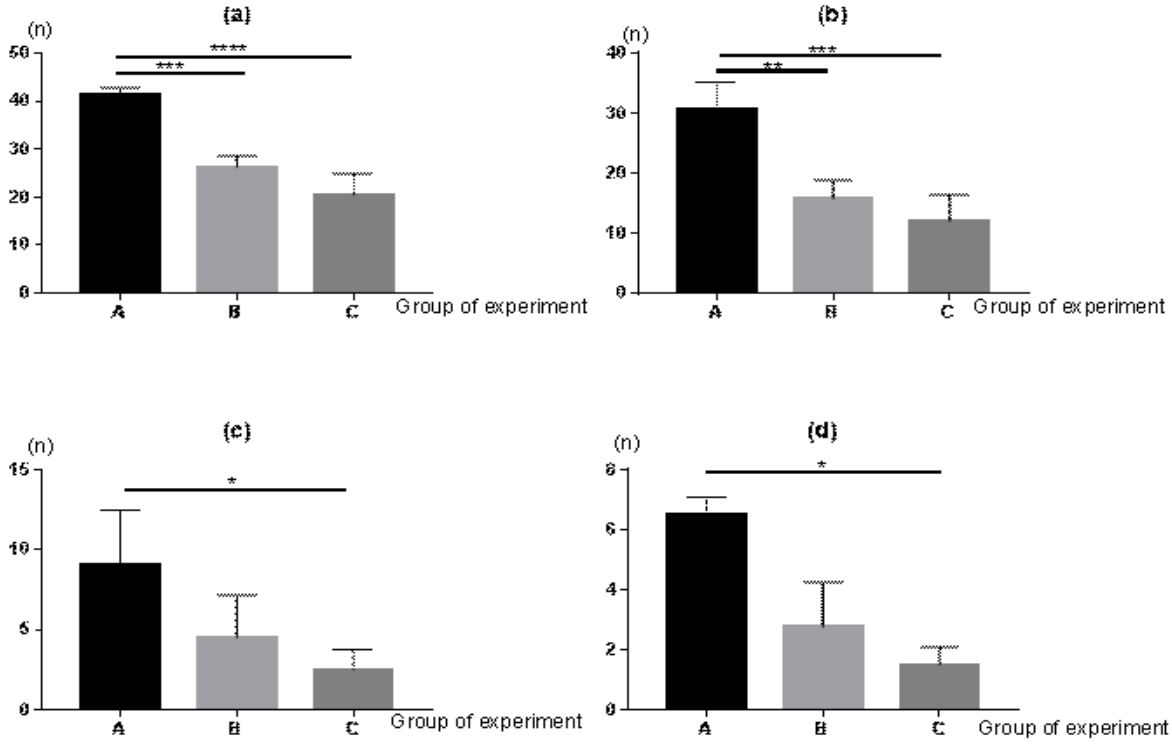

Figure 3.The effect of 20 days oral administration *Imperata cylindrica* L roots extract on the mating rate of female mice; (a) The number ovulated oocytes (b) fertilized oocytes, (c) cleavage embryos, (d) blastocyst embryos. (A) control group, (B) 90 mg/kg b.w, (C) 115 mg/kg b.w.

active compounds, and those embryos could not reach preimplantation development until the blastula phase (Widiana and Sumarmin, 2016).

Phytochemical studies in the roots of *I. cylindrica var. major* resulted mostly phenolic compounds such as flavonoids, simple phenols, phenolic acids, coumarins, lignans, terpenoids, tannins, saponins, alkaloids (Liu *et al*., 2012). Flavonoids, coumarins, lignans, terpenoids, saponins, and alkaloids have phytoestrogens effects that can mimic or interfere with the action of estrogens (Grippo *et al*., 2007; Mbemya *et al*., 2017). Phytoestrogen is a natural compound contained in plants that able to bind estrogen receptor and stimulate estrogen-dependent transcription. Although the activity is low (1/100 than natural estrogen, E2), their utilization exhibits significant effect in gonadal organs (Ye *et al*., 2016; Puranik *et al*., 2019). Alkaloid inhibits implantation and has an abortifacient effect by increasing uterine contraction. They competitively bind estrogen receptor then inhibit LH and FSH secretion. In addition, flavonoid act as cell cycle modulator which can inhibit cell proliferation induce apoptosis. This implies that the failure of decidua formation leads to the failure of blastocyst implantation (Yakubu *et al*., 2010). Saponins as steroid compounds harm animal reproduction known as an abortifacient, zygote development inhibition, and antiimplantation (Francis *et al*., 2002). Saponins have cytotoxic effects specifically in developing cell example in the process of oogenesis. These estrogenic effects from *I. cylindrica* L. can interfere ovulation process, fertilization, and the development of the embryos.

This study's result is consistent with previous findings of several plants that contain phytoestrogen properties with anti-implantation and antifertility effects. Rat treated with *Anethum graveolens* L. or dill leaf and seed extract showed increased progesterone level, prolonged diestrus phase, and the disturbing regular estrous cycle. The mating was delayed and the number of live fetuses decreased compared to the control group (Monsefi, 2014). The consumption of high dose *Zingiber officinale* (ginger) decreases estrus cycle duration, implantation sites, and the number of live

fetuses. The progesterone production was impaired by disrupting corpus luteum development that needs in early pregnancy (Elmazoudy 2018). Administration of *Ginkgo biloba* to female rats induces vaginal bleeding, abortion, preimplantation and postimplantation loss, and the increasing number of non-viable fetuses (Elmazoudy, 2012).

CONCLUSION

To conclude, gavage of *I. cylindrica* L ethanolic root extract could affect mice mating rate, the development of mice embryo's cells, reduce conception and pregnancy.

SUGGESTION

Further study is required to know the minimal dose of CGG extract that causes impairment of postimplantation embryo development, abortifacient rate, and teratogenic effect towards the fetus.

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