International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304
Vol.8, No.5, pp 879-888, 2015

The Effect of Ginger Extract (*Zingiber officinale* Roscoe) on Male Leydig Cell and Testosterone Level in Carbofuran-induced Rat

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Abstract: To evaluate the protective effects of *Zingiber officinalle rosco* (Ginger) against carbofuran-induced reproductive toxicity in rats and to study the mechanisms of these effects.
Methods: Twenty five male rats were divided into five groups, each representing as A: control negative (normal saline), B: control positive (0.8 mg of carbofuran 10%), C: first experimental dose (0.8 mg of carbofuran and 500 mg of ginger extract), D: second experimental dose (0.8 mg of carbofuran (10%) and 750 mg of ginger extract), E: third experimental dose (0.8 mg of carbofuran and 1000 mg ginger extract). The research was done in one month.
Results: Treatments with only carbofuran showed a significant decrease of testosterone and increase in proliferation of leydig cell, while the combination of ginger extract with carbofuran showed a significant increase of testosterone and decrease in proliferation of leydig cell (similar results to that of the control negative group: $P>0.05$).
Conclusion: According to the obtained results, it can conclude that the extract of ginger (*Z. Officinale*) at 750 mg dose produced counteract effect to the deleterious effect on reproductive parameter (testosterone level, leydig cell histopathology) which produced by carbofuran and it is potential for therapeutic role.
Key word: carbofuran, Ginger extract, testosterone, leydig cell.

Introduction

The increasing utilization of pesticides in agriculture and local activities for controlling pests is vastly polluting the environment day after day[1]. Moreover, toxicity of the origin pesticides and their degradation products makes these chemicals become potentially dangerous. The utilization of pesticide has extremely increased during the past few years[2] despite their well-known risks for human health[3,4]. Carbofuran, the most commonly used for broad-spectrum insecticide, has been shown to cause toxicity to mammalian systems. Several epidemiological studies suggested that carbofuran delayed the neuropathy and acetyl cholinest change erase (AChE) inhibition in humans[5,6,7,8]. Insecticides may lead directly damage to spermatozoa, change the function of Sertoli cell, Leydig cell, and hormonal regulation (hormone synthesis, release, storage, transport, and clearance; receptor ligand recognition; function of thyroid; and the central nervous system)[9]. Carbofuran exposure increase neurochemical spectrum, neurobehavioral deficits, and neurophysiological[10,11,12]. The use of

carbofuran can disrupt the membrane intregity and its function[13]. It changed the levels of epinephrine, neurotransmitter-like gamma aminobutyric acid, norepinephrine, and dopamine significantly. Carbofuran can cause the disfunction of mitochondrial, denditic and neural damage in rodents[10,14]. Ram et al., (2001) indicated that long-term exposure of low concentration carbofuran can cause retardation of gonadal development which might have been mediated by the impairment of hypothalamo-neurohypophyseal-gonadal axis in this species[15]. Carbofuran exposure decreased cell proliferation, neurogenesis, and neuronal survival, alters neuronal and glial differentiation. It could lead to hypothalamic impairment[16]. Carbofuran (Furadan) has the potential to cause damage to the reproductive system through prolonged exposure. The effects including the decrease of testosterone serum concentration and increasing the proliferation of leydig cells number[17]. According to the available information about mechanism of oxidative stress caused by carbofuran indicated that the inhibition in AChE activity because carbofuran can increase the concentration of AChE at the synaptic junctions. It can cause hyperexcitation and affect to ATP and oxygen consuption in the muscle and brain. This metabolic stress in tissue can generate oxidative stress due to reactive oxigen species (ROS) production[18,19].

Ginger extract has recently been shown to have a variety of biological activities, including anticancer, anti-oxidation, anti-inflammation and anti-microbial properties[20,21,22]. Ginger have hypocholesterolaemic effects and can decrease body weight, glucose, cholesterol and alkaline phosphatase in adult male rats[23]. All Ginger's major active ingredients, such as zingerone, gingerdiol, zingibrene, gingerols and shogaols, were known to possess anti-oxidant activities[24]. Ginger was also found to possess a protective against DNA damage induced by H2O2 and enhanced sperm healthy parameters in rats[25].

Materials and methods

Animals

Adult male Wistar albino rats that have weighted 100-150g and aged 2-3 months were used in this study. The animals got standard diet, water and were kept at 25 ± 1°C. The animals were divided randomly into five groups; each group consisted of five rats.

Adult male rat (n=25), were taken from the pharmacological laboratory in the medical faculty of Brawijaya University in Malang. The animals were divided into five groups as A, B, C, D and E each comprising of five animals. Five animals of each subgroup were kept in one cage. Group A (-ve control) were given normal saline orally for a period of one month. The experimental animals of group C were given the combination of 0.8 mg/Kg Bw carbofuran with 500 mg/Kg BW ginger extract for 30 days, group D were given the combination of 0.8 mg/Kg Bw carbofuran with 750 mg/Kg Bw ginger extract for 30 days and group D were given the combination of 0.8 mg/Kg Bw carbofuran with 1000 mg/Kg Bw ginger extract for 30 days.

Preparation of ginger ethanolic extract

Ginger (*Z. officinale* Roscoe) rhizomes were purchased from (Dinas Kesehatan Propinsi Jawa Timur – Materia Medica) Batu city – Jawa Timur Indonesia. One kilogram fresh ginger rhizome was cleaned, washed under running tap water, cut into small pieces, air dried and powdered. 100 g of this powder were macerated in 1000 ml of ethanol for 12 h at room temperature and then filtered. The extract concentration were 500 mg/ml, 750 mg and 1000 mg/ml. Each animal was orally given 1 ml of the final aqueous extract[22].

Chemical

Carbofuran; Furadan 10% [2, 3-dihydro -2, 2-dimethyl-7-benzofuranyl methyl carbamate]- Were taken from pharmacology laboratory of Brawijaya University, Indonesia.

Blood Sampling

Immediately after sacrificing, the blood was collected from each animal. The blood was left to coagulate then centrifuged to separate the serum at 3000 rpm for 30 minutes. The separated serum was stored at -20°C for further analyses.

Testoterone Serum Measurement

Elabscience Elisa kit (ELISA EIA-1559, 96 Wells kit, Elabscience Instruments, Wuhan, China) was used for the determination of serum testosterone by following the analytical protocol supplied by the kit manufacturer.

Histological Examinations

Samples from the testes of the four groups were processed histologically for paraffin sections. 5-7 µm sections were prepared and stained by hematoxylin and eosin stain according to Fisher–Rasmussen [20].

Statistical Analysis

The results were expressed as mean ± SD of different groups. The differences between the mean values were evaluated by ANOVA followed by Tukey's advanced test using windows version 12.0 at the trusted rate 95 % ($\alpha < 0.05$) and probability rate of ($p< 0.05$).

Results and Discussion

The Effect of ginger extract on leydig cell number

The ginger benefit effect on testicular toxicity of carbofuran were tested in male wistar rat. To our knowledge, this is the first study that evaluates the protective effects of plant extract against testicular damage induced by carbofuran in experimental animals.

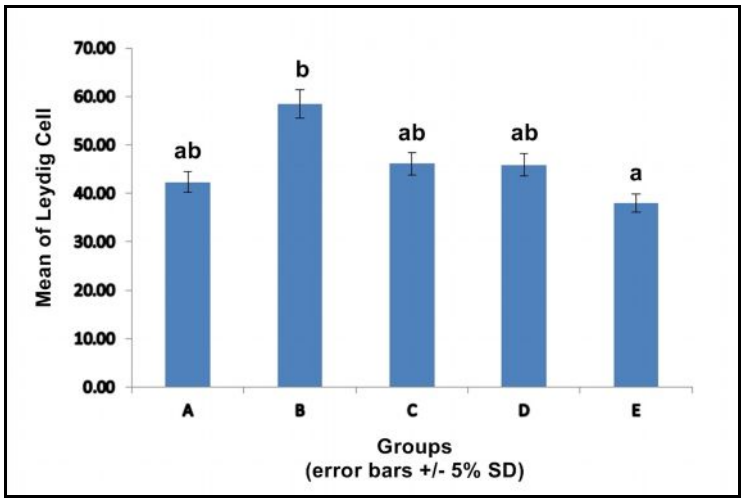

Figure 1. Bar Graph of mean leydig cell for each group; (A) Control (-): control group with normal saline, (B) Control (+): carbofuran with doses 0.8 mg/kg.bw/day; (C) Carbofuran with doses 0.8 mg/kg.bw/day and ginger 500mg/kg.bw/day; (D) Carbofuran with doses 0.8 mg/kg.bw/day and ginger 750mg/kg.bw/day; (E) Carbofuran with doses 0.8 mg/kg.bw/day and ginger 1000mg/kg.bw/day

The obtained results in the present study show that carbofuran induced many histological changes in testicular tissue of rat and cured by ginger extract. Many studies have described the decreasing effect of carbofuran on testosterone serum concentration and as well as its increasing effect on proliferation leydig cell[4,17]. Long-term exposure of carbofuran can alterate histologial of reproduction oragan in male such as leydig cells[26].

The different doses of ginger Ethanolic extract has different effects on Leydig cell (Figure 2). The effect of ginger Ethanolic extract is starting to appear when the number of Leydig cell in rats induced carbofuran becomes lower, after the treatment was given in the form of ginger Ethanolic extract started at a dose of 500 mg, compared with Leydig cell in the positive control group. Then the Leydig cell decreased when given higher doses. Thus, based on the assessment descriptively according to the mean Leydig cell, it can be said that the administration of treatment in the form of ginger Ethanolic extract at a dose of 500 mg, 750 mg and

1000 mg show different influences, where the higher dose of ginger Ethanolic extract will further lowering the Leydig cell.

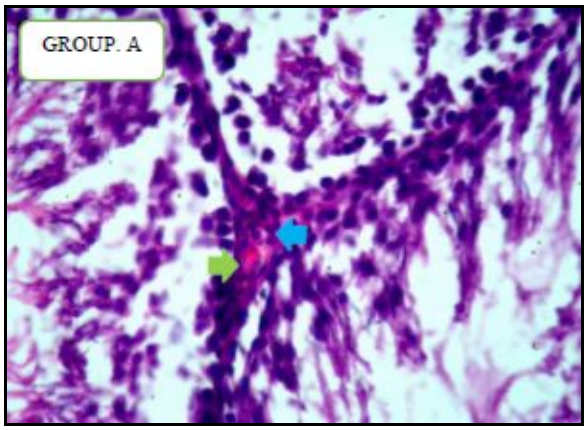

Figure 2. Normal leydig cell (H.E stain, 400x); Blue arrow = leydig cell, green arrow =blood vessel and red arrow = fibroblast cell.

Group A (negative control) rat showing the leydig cells in the microscope without treatment, where the leydig cell was observed in normal number. The figure below shows the effect of carbofuran on leydig cell.

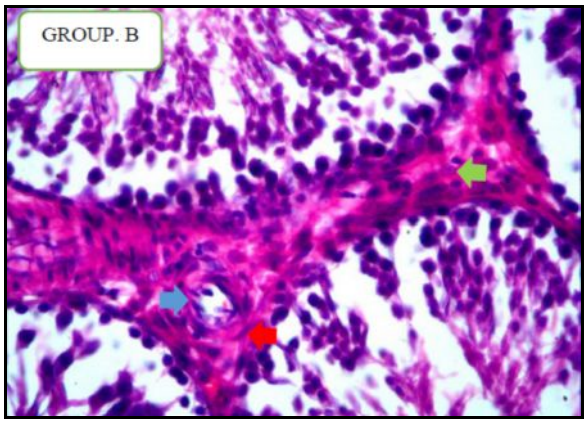

Figure 3. Effect of dose carbofuran in 0.8 mg/kg.bw/day on leydig cell proliferation (H.E stain, 400x); Blue arrow = leydig cell, green arrow = blood vessel and red arrow = fibroblast cell.

Group B (positive control) rat after treatment by 0.8 mg/kg.bw carbofuran shows an increasing in leydig cells number compared with (Figure 2) of leydig cells without treatment.

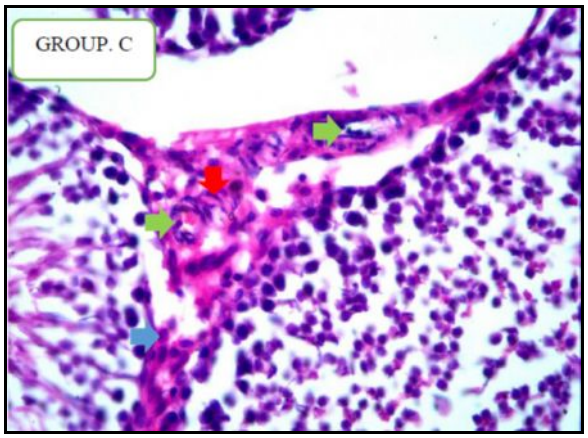

Figure 4. Effect of dose carbofuran 0.8 mg/kg.bw/day and ginger extract 500mg/kg.bw/day on leydig cell (H.E stain, 400x); Blue arrow = leydig cell, green arrow =blood vessel and red arrow = fibroblast cell.

Group C rat after treatment by 500 mg/kg.bw ginger and 0.8 mg/kg.bw/day carbofuran, show a changing in the leydig cell number which lead on the decreasing of leydig cell proliferation if compared with (figure 3) of leydig cells of group B.

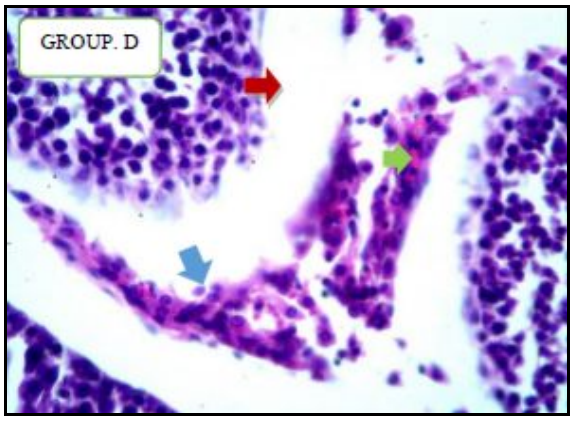

Figure 5. Effect of dose carbofuran 0.8 mg/kg.bw/day & ginger extract 750mg/kg.bw/day on leydig cell (H.E stain, 400x); Blue arrow = leydig cell, brown arrow = interstitial space, green arrow = blood vessel.

Group D rat after treatment by 750 mg/kg.bw/day ginger and 0.8 mg/kg.bw/day carbofuran, show a changing in the leydig cell number which lead on the more decreasing of leydig cell proliferation compared with (figure 4) of leydig cells of group C.

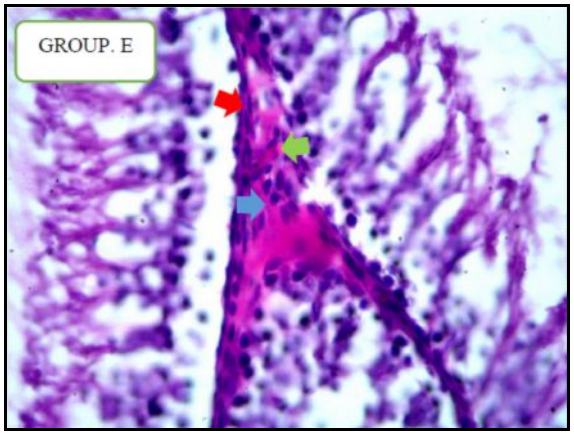

Figure 6. Effect of dose carbofuran 0.8 mg/kg.bw/day & ginger extract 1000mg/kg.bw/day on leydig cell (H.E stain, 400x); Blue arrow = leydig cell, green arrow =blood vessel and red arrow = fibroblast cell.

Group E rat after treatment by 1000 mg/kg.bw/day ginger and 0.8 mg/kg.bw/day carbofuran, show a changing in the leydig cell number which lead to more decrease of leydig cell proliferation compared with figure 5 of leydig cells of group D. In Leydig cell examination, we notes that the fifth group, treated by 0.8 mg of carbofuran (10%) and 1000 mg of ginger extract has the highest rate of effect compared to other groups, followed by the fourth group, and third group when observed in microscope. This indicates that ginger extract at fifth dose led to high decrease of leydig cell number.

In Testosterone measurement, we note that the fifth group, treated by 0.8 mg of carbofuran (10%) and 1000 mg of ginger extract has the highest rate of effect compared to other groups, followed by the fourth group, and the third group. This indicates that ginger extract at fifth dose led to high increase on serum concentration of testosterone. Testosterone involved in the development of sperm cells and derangement results widely in leydig cell dysfunction and testicular steroidogenic disorder[27].

The Effect of ginger extract on the concentration of Testosterone serum

Production of testosteron by leydig cell is main factor in spermatogenesis, it stimulated by FSH and LH under the control of hypothalamic–pituitary–testis axis[28,29]. The synthesis of sertoli cell is induced by FSH. An

androgen binding protein is needed to maintain testosterone level. Production of testosteron is induced by LH with interstitial cells of the testis[28]. Gonadotropins and testosterone are the main controllers of germ cell development. The Development of germ cell is depend on the balanced endocrine interplay of hypothalamus, pituitary and testis[30]. Carbofuran can change hormonal balance by its toxic effect or possibly through the alteration of neuroendocrine environment resulting in inhibition of acetyl cholinestase[28].

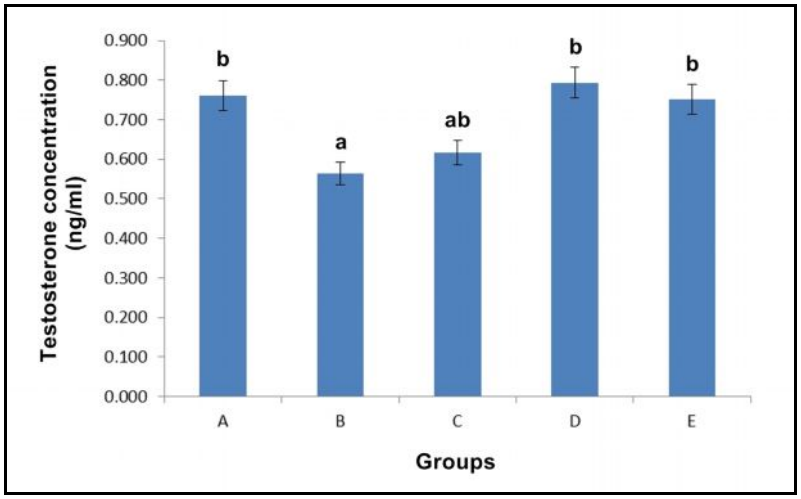

Figure 7. Bar Graph of mean of testosterone in rat serum for each group; (A) Control (-): control group with normal saline; (B) Control (+): carbofuran with doses 0.8 mg/kg.bw/day; (C) Carbofuran with doses 0.8 mg/kg.bw/day and ginger 500mg/kg.bw/day; (D) Carbofuran with doses 0.8mg/kg.bw/day and ginger 750mg/kg.bw/day; (E) Carbofuran with doses 0.8mg/kg.bw/day and ginger 1000mg/kg.bw/day

The different doses of ginger Ethanolic extract influence or give different effects on testosterone concentration (Figure 7). The effect of ginger Ethanolic extract is starting to appear when the level testosterone concentration in rats induced carbofuran becomes higher, after the treatment was given in the form of ginger Ethanolic extract started at a dose of 500 mg, compared with the testosterone concentration in the positive control group. Then the testosterone concentration increased when given higher doses. Thus, based on the assessment descriptively according to the mean concentration of testosterone, it can be conclude that the administration of treatment in the form of ginger Ethanolic extract at a dose of 500 mg, 750 mg and 1000 mg show different influences, where the 750 mg dose of ginger Ethanolic extract will further increase the concentration of testosterone.

Based on the results of analysis of variance for testosterone concentration, the data show a significance value of 0.001 ($p < 0.05$), so that Ho is rejected, and it can be concluded that there are differences in the testosterone concentration data in each treatment group of ginger ethanolic extract.

This study show that carbofuran can induce an increase of Leydig cells numbers associated with decrease in testosterone level. The decrease of testosterone biosynthesis together with increased leydig cells numbers are apparently due to LH induction of aromatase activity in Leydig cells. Disorders in testicular steroidogenesis and Leydig cell proliferation have also been reported in transgenic rat overexpressing human chorionic gonadotropin, an analogue of LH Leydig's cell proliferation, is thought to be due to a faulty hypothalamic-pituitary-testicular axis with resultant chronic Leydig's cell stimulation. It is because normal Leydig's cells produce androgens as a result of luteinizing hormone stimulation. A Decrease in testosterone level affect to increased serum luteinizing hormone in Leydig's cell proliferation. Human chorionic gonadotropin is structurally similar to luteinizing hormone and can also causes Leydig's cell proliferation[31].The increase in leydig cell proliferation may be caused by increasing testicular steroidogenesis as result of a lowered testosterone level[32]. Based on Leydig cell development, two ways of proliferation was revealed:that are; dependent and independent LH. Development of leydig cell is related to gene expression[33].

It was reported that most of the pesticides exerted its toxic actions through formation of free radicals. Free radicals could cause structural abnormalities, some degenerative alterations in reproductive system, and cross linkage of the nucleic acids[34]. The testicular changes recorded in the present study might be due to free

radical reactions.

Ginger extracts have been extensively studied for a broad range of biological activities, especially antioxidant activities[35] found that ginger significantly lowered lipid peroxidation by maintaining the activities of the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxides in rats. Ginger is a strong antioxidant substance and may be prevent the free radicals generation. It is potential as a safe herbal medicine with few and insignificant adverse/side effects[36]. Ginger rhizome contains a wide variety of both antioxidative[37] and androgenic activity[22]. Increaded testosterone level may be related to Leydig cells which known in the secret testosterone. It can be known by the higher number of leydig cell, the higher level of testosteron. It has been widely known if ginger has antioxidant properties such as zingerone, gingerdiole, zingirbrene, gingerls and shagaols. These compounds protect from DNA damage that induced by H_2O_2[24,38]. Ginger extract is manifested a very good scavenging of 2,2-Diphenyl-1-picril hydrazyl radical (DPPH) and reduced its reducing capacity. The extract can be used as an antioxidant at an earlier stage of fat oxidation. The ginger extract shows an antioxidant activity compare with that of butylated hydroxytoluene (BHT) in inhibiting the lipid peroxidation. The ginger extract also shows an inhibiting effect with regard to the hydroxyl radicals, better than that of quercetin. Under the conditions of conducted experiments the polyphenols in the ginger extract also demonstrates a higher chelatoforming capacity with regard to Fe3+, leading to the prevention of hydroxyl radicals initiation which are known as inducers of lipid peroxidation[39].

Discussion

The ginger benefit effect on testicular toxicity of carbofuran were tested in male wistar rat. To our knowledge, this is the first study that evaluates the protective effects of plant extract against testicular damage induced by carbofuran in experimental animals.

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Conclusion

The findings of this study suggests that ginger extract could attenuate reproductive system toxicity by increasing the testosterone level as well as decreasing the leydig cell number in carbofuran treated rat. So, according to the obtained results and the increasing of testosterone concentration and the decreasing of leydig cells number, it can conclude that ginger extract at 1000 mg dose produced effect that counteract the deleterious effect on reproductive parameter (testosterone level, leydig cell histopathology) produced by carbofuran and therefore, the combination of ginger with carbofuran may play a beneficial therapeutic role. This indicates that ginger may be considered safe for future human consumption, effective supplementary drug for sexual activity and fertility improvement as evident at least from the results of our experiments.

Acknowledgment

We would like to thank to the Pharmacology Laboratory, Medical Faculty, Brawijaya University for supporting this research project.

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