Effect of clove flower extract (*Syzygium aromaticum*) on spermatogenic cells and Leydig cells in the unilateral cryptorchidism albino rat model (*Rattus norvegicus*)

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Abstract

The increase in testicular temperature caused by cryptorchidism can lead to elevated production of reactive oxygen species (ROS), lipid peroxidation, and a decrease in antioxidant enzymes such as catalase, glutathione peroxidase, and others. Clove (*Syzygium aromaticum*) contains eugenol, a compound known for its potent antioxidant properties. This study aimed to evaluate the effect of clove flower extract on spermatogenic cells (spermatogonia, primary spermatocytes, and spermatids) and Leydig cells in albino rats (*Rattus norvegicus*) with surgically induced cryptorchidism. The method used was posttest-only control group design. A total of 24 male rats were randomly assigned to six groups, divided into two treatment durations of 18 days and 36 days. The 18-day interval groups consisted of K-1, K+1, and P+1, while the 36-day interval groups included K-2, K+2, and P+2. One-way ANOVA was used to analyze the number of spermatogonia, primary spermatocytes, spermatids, and Leydig cells. Duncan's multiple range test was applied to data showing significant differences ($P < 0.05$). The results indicated that administration of clove flower extract in albino rats with surgically induced cryptorchidism, at both 18-day and 36-day intervals, significantly increased the number of spermatogenic cells (spermatogonia, primary spermatocytes, and spermatids) and Leydig cells. Administration of clove flower extract significantly increased the number of spermatogenic and Leydig cells in albino rats with surgically induced cryptorchidism, indicating its potential as a supportive therapy against oxidative stress-induced testicular damage. Clove flower extract may serve as a temporary supportive therapy in unilateral cryptorchidism cases; however, surgical intervention remains strongly recommended.

Eugenol, reproductive health, testicular conditions

Cryptorchidism is a condition in which one or both testes fail to descend into the scrotum (Khan et al. 2018). Cryptorchidism is one of the most common congenital abnormalities observed in animals (Mahiddine and Kim 2021). Small ruminants are more prone to developing unilateral cryptorchidism, which commonly affects the right lateral testis (Oguejiofor et al. 2018).

Cryptorchidism was among the most common congenital disorders in dogs, with reported incidence rates ranging from 1.2% to 10% (Gradil and McCarthy 2023). Small breed dogs such as the Cairn Terrier, English Bulldog, Pomeranian, Pekingese, Old English Sheepdog, Shetland Sheepdog, Toy Poodle, Miniature Schnauzer, Maltese, Miniature Poodle, and Boxer are more susceptible to cryptorchidism than large breed dogs (Spangenberg 2021). Cases of cryptorchidism have also been reported in wild felids such as the Amur leopard (*Panthera pardus orientalis*) (Napier et al. 2018) and the jaguar (*Panthera onca*) (Jorge-Neto et al. 2020).

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Cryptorchidism could disrupt the epithelial cycle of the seminiferous tubules, reduce testicular weight, and impair spermatogenesis, which is characterized by the degeneration of spermatogenic cells (Zheng et al. 2019). Cryptorchidism could also alter the morphology of Leydig and Sertoli cells (Murphy and O'Shaughnessy 1991). The elevated testicular temperature in cryptorchid individuals induces oxidative stress responses in germ cells, promotes the formation of sperm with abnormal morphology, damages sperm DNA integrity, and disrupts the regulation of physiological homeostasis (Gao et al. 2022).

Testicular tissue contains unsaturated fatty acids and has low antioxidant levels, making it highly susceptible to reactive oxygen species (ROS), which oxidize lipids, proteins, and deoxyribonucleic acid (DNA), ultimately leading to cellular damage. Maintaining a balance between the production and neutralization of ROS is crucial for preserving normal physiological functions in the body (Tekayev et al. 2019). The integrity of the blood-testis barrier in cryptorchid testes is compromised due to elevated temperatures. Elevated scrotal temperatures lead to excessive ROS production, lipid peroxidation, and reduced levels of antioxidant enzymes such as glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT), which can result in infertility and germ cell death (Munyali et al. 2020).

Lipid peroxidation was a chain reaction triggered by free radicals, particularly ROS, binding to polyunsaturated fatty acids (PUFAs). Malondialdehyde (MDA) was the final product formed during the lipid peroxidation process (Ayala et al. 2014). Lipid peroxidation could occur when ROS, acting as oxidizing agents, interact with cellular membranes (Yadav et al. 2019). Excessive levels of ROS could damage the integrity of DNA in the sperm nucleus and reduce the fluidity of the sperm plasma membrane, ultimately leading to impaired spermatogenesis. Cryptorchidism may result in pathological forms of necrosis. Necrosis associated with cryptorchidism has been demonstrated through various molecular and morphological analyses in multiple animal models (Dündar et al. 2005).

Antioxidants play a crucial role in neutralizing free radicals and inhibiting the formation of ROS (Adwas et al. 2019). Oxidative stress arises from an imbalance between the antioxidant defense system and the production of ROS. Nucleic acids, proteins, and lipids in tissues can be damaged when exposed to excessive levels of ROS (Avci et al. 2019). Clove flowers contain various phenolic compounds, including gallic acid, eugenol, β-caryophyllene, eugenol acetate, and α-humulene. These compounds possess potential applications in the fields of cosmetics, pharmaceuticals, agriculture, and food industries (Rani and Jena 2021). Eugenol exhibits antioxidant activity in testicular tissue (Ekinci et al. 2019), protects the activity of GSH-Px, CAT, and SOD, and contributes to the reduction of MDA levels in the testes (Han et al. 2019). Eugenol belongs to the phenolic class of antioxidants. It is considered a potent antioxidant, reportedly five times more effective than vitamin E in preventing oxidative damage caused by free radicals (Nagababu et al. 2010).

Administration of eugenol contained in clove flower (*Syzygium aromaticum*) can reduce oxidative stress and improve testicular tissue damage in animals with cryptorchidism by increasing the number of spermatogenic cells (spermatogonia, primary spermatocytes, and spermatids), as well as Leydig cells. This study aimed to evaluate the effect of clove flower extract on spermatogenic cells (spermatogonia, primary spermatocytes, and spermatids) and Leydig cells in albino rats (*Rattus norvegicus*) with surgically induced cryptorchidism.

Materials and Methods

Ethical approval

This research received ethical clearance No. 1.KEH.011.01.2022, issued by the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga.

Animals and study design

This experiment involved 24 male rats aged 15 to 17 days, using a posttest-only control group design. The rats were randomly assigned to six experimental groups. The groups included: (1) the negative control group

(K-1 and K-2), which received sham surgery and aquadest for 18 and 36 days, respectively; (2) the positive control group (K+1 and K+2), which underwent surgical induction of cryptorchidism followed by aquadest administration for 18 and 36 days, respectively; and (3) the treatment group (P+1 and P+2), which underwent surgical induction of cryptorchidism followed by clove flower extract administration for 18 and 36 days, respectively.

Surgical induction of cryptorchidism was performed following the method described by Tekayev et al. (2019), beginning with anaesthesia using a mixture of 3.75 ml ketamine, 5.75 ml distilled water, and 0.5 ml xylazine. The anaesthetic mixture was administered intraperitoneally at a dose of 0.2 ml per 100 g of body weight. After anaesthesia was completed, a small incision was made in the inguinal region, on the right side near the gubernaculum. The external inguinal ring was used to separate the right gubernaculum from surrounding tissues before repositioning it into the abdominal cavity. The external inguinal canal was sutured to prevent the testis from descending. In the unilateral cryptorchidism model, only one testis was surgically induced, while the contralateral testis was left intact to serve as a reference for evaluating post-surgical changes.

Following the completion of the surgical procedure, the rats were returned to their cages. A sham operation was performed on rats in the negative control group by making an incision in the right inguinal region near the gubernaculum. The right gubernaculum was then isolated and gently manipulated by pushing it into the abdominal cavity to simulate the cryptorchidism induction procedure. After the manipulation was completed, the right gubernaculum was repositioned to its original location. The final stage of the sham operation involved closing the incision with sutures (Tekayev et al. 2019).

Euthanasia of the experimental animals was performed using the cervical dislocation method on days 18 and 36 after surgical induction of cryptorchidism. The right and left testes were collected using surgical instruments and then immersed in 10% buffered formalin for histopathological processing. Spermatogenic cells in the seminiferous tubules were observed microscopically using a trinocular microscope at × 400 magnification. Spermatogenic cells were counted in five seminiferous tubules from each treatment group and replicate.

Statistical analysis

Normality test showed that the data were normally distributed, and homogeneity test confirmed that the data were homogeneous for spermatogenic cells. Subsequently, spermatogenic cell data were analysed using ANOVA; if significant differences were found ($P \leq 0.05$), Duncan's test was applied for *post hoc* analysis.

Results

Table 1 shows that the average number of spermatogenic cells was lower in the positive control groups (K+1 and K+2) compared to the negative control groups (K-1 and K-2). This decrease was directly correlated with a reduction in the average number of Leydig cells in the positive control groups. Furthermore, the number of spermatogenic and Leydig cells increased in the P+1 and P+2 treatment groups, with values in the P+2 group approaching those observed in the negative control groups (K-1 and K-2). According to Duncan's test, the average numbers of spermatogenic and Leydig cells differed significantly ($P \leq 0.05$) among the groups.

Table 1. The number of rat spermatogenic cells in the control and treatment groups (mean ± standard deviation).

18-day interval group				
Treatment	Spermatogonia	Primary spermatocyte	Spermatids	Leydig cells
K-1	$36.00^{b} \pm 2.16$	$27.75^{b} \pm 0.95$	$6.25^{b} \pm 1.25$	$39.50^{b} \pm 3.10$
K+1	$23.75^{a} \pm 3.86$	$13.25^{a} \pm 2.50$	$2.00^{a} \pm 1.63$	$20.75^{a} \pm 1.89$
P+1	$31.00^{b} \pm 4.83$	$24.75^{b} \pm 1.89$	$4.25^{b} \pm 0.95$	$38.25^{b} \pm 1.25$
36-day interval group				
Treatment	Spermatogonia	Primary spermatocyte	Spermatids	Leydig cells
K-2	$35.50^{b} \pm 2.64$	$33.00^{b} \pm 2.58$	$5.00^{b} \pm 0.81$	$24.00^{b} \pm 2.58$
K+2	$23.50^{a} \pm 1.91$	$13.00^{a} \pm 2.58$	$1.75^{a} \pm 0.95$	$13.00^{a} \pm 1.41$
P+2	$33.50^{b} \pm 2.08$	$31.50^{b} \pm 2.64$	$4.00^{b} \pm 0.81$	$22.00^{b} \pm 2.16$

[a,b]Different superscripts in columns show significant differences ($P \leq 0.05$). K-1: Negative control (sham and distilled water operation for 18 days); K+1: Positive control (cryptorchidism and aquadest for 18 days); P+1 (cryptorchidism and clove flower extract 70 mg/kg BW for 18 days); K-2: Negative control (sham and distilled water operation for 36 days); K+2: Positive control (cryptorchidism and aquadest for 36 days); P+2 (cryptorchidism and clove flower extract 70 mg/kg BW for 36 days).

All treatment groups showed significant differences ($P \leq 0.05$), as indicated by Duncan's test. This was demonstrated by the substantial reduction in Leydig and spermatogenic cells in the positive control groups (K+1 and K+2). In contrast, the number of spermatogenic and Leydig cells significantly increased in the P+1 and P+2 treatment groups (Plate V, Fig. 1).

Discussion

Spermatogenesis is the process of forming spermatozoa and is considered a highly active process, capable of producing approximately 1,000 spermatozoa per second. The high rate of cell division suggests a high mitochondrial oxygen demand in the embryonic epithelium of the testis (Gandhi et al. 2017). The testis is an organ highly sensitive to free radicals. Free radicals can damage the spermatozoa membrane and disrupt the process of spermatogenesis. Spermatogenic cell membranes contain PUFAs, which are easily targeted by free radicals, leading to lipid peroxidation reactions (Boubdallah et al. 2022).

Cryptorchidism, also known as undescended testis, is a condition in which one or both testicles fail to descend into the scrotum, affecting male reproductive development. The scrotum provides an optimal temperature environment for spermatogenesis (Kumar et al. 2012). Testicles retained in the abdominal cavity are exposed to higher temperatures, which can damage germ cells and the seminiferous epithelium, ultimately disrupting the spermatogenesis process (Liu et al. 2012). Primary spermatocytes and spermatids are heat-sensitive due to the expression of thermosensitive proteins such as HTRA2 (Hayashi et al. 2006), temperature-required serine protease (TRS1) (Han et al. 2003), and HSP105 (Kumagai et al. 2000). Elevated intra-abdominal temperatures in cryptorchidism induce excessive ROS production, which increases oxidative stress (Ko et al. 2014). ROS hyperactivity in the testes leads to increased lipid peroxidation of the spermatozoa plasma membrane, impairing sperm capacitation (Guasti et al. 2017). Oxidative stress in the testes triggers apoptosis of spermatogenic cells, explaining the mechanism underlying azoospermia in cryptorchidism (Kobayashi et al. 2013).

Cryptorchidism induction without therapeutic intervention in the K+1 and K+2 treatment groups resulted in a significant average reduction in both spermatogenic and Leydig cells in the experimental animals. This finding is consistent with the statement by Huff et al. (1993), who reported that in cases of cryptorchidism, there is a reduction in the number of Leydig cells and adult dark spermatogonia due to delayed formation, which subsequently leads to a decrease in the development of primary spermatocytes.

Clove flower extract exhibits high antioxidant activity, which is attributed to the interaction between eugenol and minor constituents present in the extract. The antioxidant mechanism of eugenol involves three pathways: hydrogen atom donation followed by delocalization of substituted groups, dimerization between two phenoxyl radicals, and complexation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) with aryl radicals (Sokamte et al. 2016).

The average number of spermatogenic cells (spermatogonia, primary spermatocytes, and spermatids) in the P+1 and P+2 treatment groups increased as a result of the antioxidant effects of eugenol observed in this study. The P+1 treatment group, which received 70 mg/kg BW of clove flower extract for 18 days after cryptorchidism induction, and the P+2 group, which received the same dose for 36 days, showed higher average spermatogenic cell counts compared to the K+1 and K+2 groups. These results demonstrate that administration of clove flower extract in cryptorchidism-induced rats has a positive effect by preserving the number of spermatogenic cells following surgical induction. These findings are consistent with the statement by Ulanowska and Olas (2021), which reported that eugenol possesses a strong antioxidant potential. Antioxidants have also been shown to prevent germ cell death in cryptorchid testes by interfering with the necrosis process (Bergh and Söder 2007). Necrosis is a form of pathological cell death (Friedlander 2003). Necrosis can

occur when ROS is overproduced (Turan 2010). Eugenol acts as an antioxidant and can reduce ROS levels. This is supported by the findings of Ma et al. (2021) who reported that ROS levels can be reduced through eugenol therapy.

Spermatogonia showed higher average numbers compared to primary spermatocytes and spermatids. This finding is consistent with Bianchi et al. (2017), who reported that primary spermatocytes and spermatids are more susceptible to apoptosis-related cell loss in cryptorchidism, while spermatogonia are more resistant to temperature fluctuations associated with the condition (Pérez-Crespo et al. 2008).

As can be seen in Table 1 and Fig. 1, the average number of spermatogenic cells in the K+1 treatment group was higher than in the K+2 group. The results observed in both treatment groups are consistent with the findings of Dutta et al. (2013), who compared rats with an interval of 1.5 cycles of the seminiferous tubule epithelium to those with 2 × 1.5 cycles. Their study demonstrated that the 1.5-cycle interval was associated with relatively less testicular damage compared to the extended interval group.

Leydig cells are highly susceptible to free radicals which directly affect the hypothalamic and pituitary axes (Rizal and Fauzi 2019). An increase in free radicals leads to oxidative stress which inhibits the hypothalamic-pituitary-testicular axis. This inhibition is followed by a decrease in the secretion of follicle-stimulating hormone, luteinizing hormone, and testosterone synthesis by Leydig cells (Plunk and Richards 2020).

According to Winters et al. (2018), Leydig cell insufficiency may occur in cases of cryptorchidism. The K+1 and K+2 treatment groups showed a reduction in the average number of Leydig cells. The reduction in Leydig cell numbers observed in cryptorchidism is associated with subfertility due to impaired germ cell maturation (Winters et al. 2018).

Research by Boudou et al. (2013) reported that clove flower (*Syzygium aromaticum*) extract can significantly promote the regeneration of Leydig cells. The eugenol compound found in clove acts as a potent antioxidant for the testes by inhibiting lipid peroxidation and preventing oxidative stress-induced damage (Boudou et al. 2013). These findings are consistent with the results of the present study. The P+1 group (surgical induction of cryptorchidism followed by clove flower extract administration for 18 days) and the P+2 group (36 days of treatment) maintained higher average Leydig cell counts compared to the untreated cryptorchid groups. Therefore, eugenol treatment prior to testicular repositioning (or orchiopexy) may serve as a supportive approach to prevent further testicular damage, especially in cases where immediate surgical intervention is not possible.

Beyond its potential role as a supportive therapy for cryptorchidism, clove flower extract may also have broader applications in maintaining testicular function, particularly in breeding males. Eugenol, a potent antioxidant found in clove, may help protect germ cells and reduce oxidative stress caused by systemic diseases or adverse environmental conditions such as high ambient temperatures in tropical regions. These stressors are known to impair sperm quality, testosterone production, and antioxidant enzyme activity in the testes (Choi et al. 2014; Oroojan et al. 2020; Wang et al. 2023). Therefore, clove flower extract could be considered as a preventive or supportive measure to preserve reproductive performance in breeding males exposed to such challenges.

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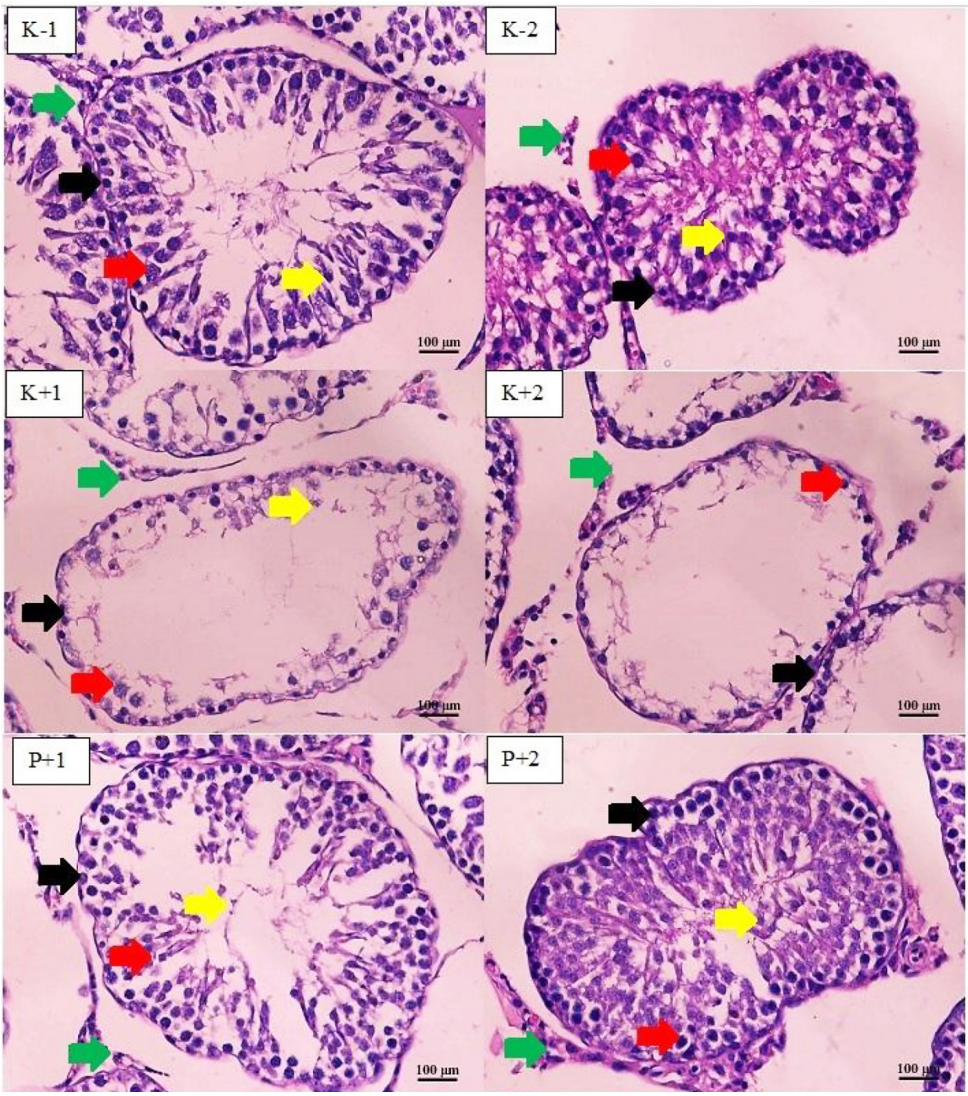

Fig. 1. Overview of microscopic images of seminiferous tubules with haematoxylin&eosin staining at a × 400 magnification (trinocular microscopy; Nicon Eclipse 200): spermatogonia cells (black arrows), primary spermatocyte cells (red arrows) spermatid cells (yellow arrows) Leydig cells (green arrows). The K+1 and K+2 treatment groups (cryptorchidism and aquadest for 18 and 36 days) showed a decrease in the number of spermatogenic cells and Leydig cells compared to the K-1 and K-2 treatment groups (sham and distilled water operation for 18 and 36 days). The P+1 and P+2 treatment groups (cryptorchidism and clove flower extract 70 mg/kg BW for 18 and 36 days) showed an increase in spermatogenic cells and Leydig cells.