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The effect of methanol extract of cloves (*Syzygium aromaticum*) on vimentin expression in the testes of rats (*Rattus norvegicus*) with induced cryptorchidism

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Abstract

Background: Clove is one of the spices that have strong antioxidant activity among other spices. The high concentration of ROS in cryptorchid testes is a major factor in the risk of developing testicular tumors. The aim of this study is that to determine the effect of methanol extract of cloves (*Syzygium aromaticum*) on the expression of Vimentin testis of white rats (*Rattus norvegicus*) with a unilateral cryptorchidism model.

Methods: The experimental animals used were 24 male rats aged 21 days which were divided into six treatment groups. The negative control group was conducted sham surgery and it was given aquadest orally for 18 days (K-1) and 36 days (K-2). Meanwhile, the positive control group was induced by cryptorchid surgery and it was given aquadest orally for 18 days (K+1) and 36 days (K+2). Moreover, the treatment group was induced by surgical induction of cryptorchidism and treated with methanol extract of cloves at a dose of 70 mg/kg BW orally for 18 day (P+1) and 36 days (P+2). Vimentin immunostaining was evaluated by using the Index Remmele Scale (IRS) scoring method. Furthermore, data analysis by using Kruskal Wallis followed by Mann Whitney test.

Results: The treatment group variables give a significant difference ($p<0.05$) on the expression of Vimentin, but no significant difference is found between the variable days of treatment and the treatment group on the expression of Vimentin.

Conclusion: The addition of cloves methanol extract can reduce the expression of Vimentin in the testes of rats with unilateral cryptorchidism model.

Introduction

Cryptorchidism is condition where failure of one or both testes to enter the scrotum. This situation is a congenital anomaly that has a reported incidence of more or less 1-10% in dogs and up to 2% in cats [1]. In human, the prevalence of boys who are born weighing more than 2.5 kg stated to be between 1.8 and 8.4%, at ages of 3 months and 1 year are respectively 0.9-1.6 and 1.0-1.5%, while at birth for boys with premature birth and/or low birth weight varies from 1.1 to 45.3% [2]. The exact cause of cryptorchidism remains unknown, but the anomaly is considered to be multifactorial. The development of the condition may be influenced by endocrine, environmental, genetic, anatomical, and mechanical variables. Several hormones such as Leydig cell-derived hormone, testosterone, and insulin-like factor 3 (INSL3) are suggested to play a role in testicular descent [3].

The high temperature in the cryptorchid model causes increased generation of *reactive oxygen species* (ROS) as a result of the testicular tissue's decreased *superoxide dismutase* (SOD) activity. The high concentration of ROS in cryptorchid testes is a major factor in the risk of developing testicular tumors [4]. Increased oxidative stress-induced ROS in several cancer cell types have been associated with the induction of epithelial mesenchymal transition (EMT), as evidenced by decreased cell adhesion-related molecules such as E-cadherin, and increased mesenchymal markers such as Vimentin [5].

Several hypotheses assume that the effect of high temperature is a predisposing factor for neoplasia of cells in the testes, especially Sertoli cell tumors [6]. Sertoli cell tumors are slow growing, non-invasive with low malignancy. However, the risk of becoming malignant increases when the tumor occurs in the testis that is retained in the abdomen [7]. Sertoli cell tumor immunoreactivity has shown that the expression of inhibin alpha and vimentin has the highest immunoreactivity among other antibodies [8].

Clove is one of the spices that have strong antioxidant activity among other spices. Clove plant is one of the major sources of phenolic complexes such as eugenol, eugenol acetate and gallic acid [9]. Eugenol as an antioxidant were attributed to a strong ability to donate hydrogen and its efficiency in scavenging of superoxide, hydrogen peroxide and other free radicals [10]. Eugenol compounds also have therapeutic potential by inhibiting EMT which can reduce the ability of cells to migrate [11].

Methods

Ethical approval

Animal Care and Use Committee Faculty of Veterinary Medicine Universitas Airlangga had granted ethical approval for this research. (number 1.KEH.011.01.2022). The United Kingdom Animal Act of 1986 governed every technique carried out for this study. All operations were performed under anaesthetic, and every attempt was made to lessen the pain.

Animals

In this research, 24 male white rats (Rattus norvegicus) strain of Sprague-dawley, aged 21 days served as the samples. K-1 and K-2 negative controls: each 4 rats underwent *sham* and was given aquadest orally for 18 days and 36 days. Positive control K+1 and K+2: 4 rats were induced by cryptorchid surgery and given orally with distilled water for 18 days and 36 days, respectively. Treatment P+1 and P+2: each 4 rats were induced by cryptorchid surgery and treated with cloves extract (*Syzigium aromaticum*) at a dose of 70 mg/kg BW orally for 18 days and 36 days.

Surgical procedure

The surgical preparation was started by anesthetizing the rats with Xylazine (100 mg/ml) and Ketamine (50mg/ml). Anesthesia was prepared by mixing 5.75 ml distilled water, 3.75 ml Ketamine, and 0.5 ml Xylazine. After that the mixture was given intraperitoneally 0.2ml/100g body weight [12]. Each rat underwent a short incision that extended to the gubernaculum in the right inguinal region. From the external inguinal ring, the right gubernaculum is separated from the tissues and forced into the abdominal cavity. The inguinal canal is closed with sutures to prevent descent of the testes. After the surgical procedure was completed, the rats were returned to the cage with their mother because they were still in the lactation period [13]. Sham is performed by manipulating the gubernaculum by pushing it into the abdominal cavity so as to perform cryptorchid induction surgery. After the manipulation is complete, the gubernaculum is returned to its original position and the incision is closed with sutures.

Methanol extract of Cloves (Syzygium aromaticum) preparation

The dried cloves samples were crushed and divided to 50 gram. A Soxhlet extractor was used to contain the sample. Methanol (90%) was placed into a Soxhlet flask. The extraction procedure went on for 6-7 hours at methanol's boiling point (65-67°C). After filtering the mixture, a rotary vacuum evaporator was used to evaporate the solvent to obtain the crude extract [14].

Collection of testis tissue

Rats from each group were sacrificed by cervical dislocation on days 18 and 36 after cryptorchid induction.

Immunohistochemical examination of Vimentin expression

Vimentin expression was observed by modified IRS-evaluation. It sums the percentage of cells in each intensity category as well as evaluating the grade of color intensity visualized. Multiplying the percentage of positive cells score (A) by the color reaction intensity score (B) yields the IRS.

A (Percentage of positive cells)	B (Intensity of Staining)
0: no positive cells	0: no colour reaction
1: <10% of positive cells	1: mild reaction
2: 11%-50% of positive cells	2: moderate reaction
3: 51%-80% of positive cells	3: intense reaction
4: >80% of positive cells	

Table 1: Semi-quantitative immunoreactive score (IRS) taking into account the percentage of stained cells (A) and the intensity of reaction product (B) in which the final results correspond to the product of the two variables (AxB).

Data Analysis

The data has been collected then analyzed using the SPSS 25 (Statical Product and Service Solutions) program by using Kruskal-Wallis test followed by Mann-Whitney test.

Results

Table 2 shows that cryptorchid induction led to an increase in the mean of Vimentin expression in the both positive control group (K+1 and K+2), which were 9.0 and 9.58, respectively. The negative control group (K-1 and K-2) had the lowest mean expression, 0.4 and 1.06, respectively. Treatment group (P+1 and P+2) were similar to those in the control group, 1.49 and 2.18, respectively.

Days	Group	Score Expression (X ±SD)
18 days	K-1	0.4^{a} ±0.255
	K+1	9.0^{c} ±2.297
	P+1	1.49^{ab} ±2.215
36 days	K-2	1.06^{ab} ±0.993
	K+2	9.58^{c} ±4.079
	P+2	2.18^{b} ±0.980

Table 2: Average score and standard deviation of Vimentin expression on rats (*Rattus norvegicus*).

Discussion

The negative control group still expressed Vimentin expression only in the apical cytoplasm of Sertoli cells because Vimentin plays a role in forming the cellular framework to protect Sertoli cells from surviving under environmental hazards [15]. Vimentin filaments are found in the perinuclear region germ cells to the seminiferous epithelium [16].

The high value of Vimentin expression in the positive control group (K+1 and K+2) was caused by the collapse and increase of Vimentin protein in Sertoli cells of cryptorchid testis. The expression of the collapse of Vimentin protein into Sertoli cells is a defensive response to heat stress that can weaken germ cell contact to Sertoli cells [15]. The increased production of ROS that surpasses the tissue's antioxidant capacity causes oxidative stress as the temperature rises in the cryptorchid testis [17].

The presence of ROS can trigger hydroxyl radicals that can break DNA chains and can damage all cell membrane systems by increasing lipid peroxidation, as well as being able to degrade proteins and oxidize proteins that can result in injury to cells [18,19]. ROS are important cellular secondary messengers in diverse biological processes in cancer cells. ROS are increased in many types of cancer and their development is involved in tumor progression by inducing epithelial mesenchymal transition (EMT). EMT is a significant factor in tumor metastasis, in which the epithelial cells lose their polarity, cell adhesion, and cell mobility [20,21].

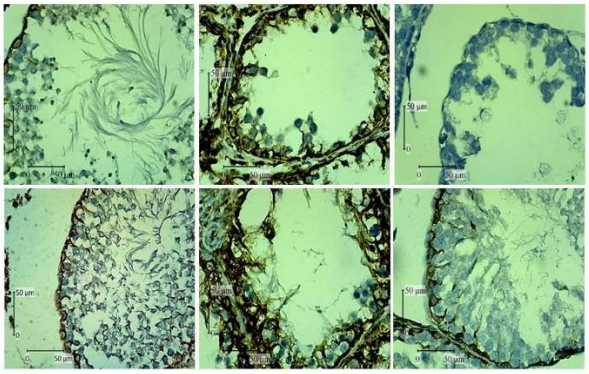

Figure 1: Imunohistochemical staining for vimentin with magnification x400 (red arrows indicate moderate intensity and yellow arrows indicate strong intensity). K-1: negative control 18th day; K+1: cryptorchid testis, 18th day; P+1: cryptorchid testis with treatment, 18th day; K-2: negative control 36h day; K+1: cryptorchid testis, 36th day; P+1: cryptorchid testis with treatment, 36th day.

Oxidative stress caused by *Hypoxia Inducible Factor-1* (HIF-1). It can control the Vimentin protein's transcription. Both natural and induced cryptorchidism showed an increase in HIF-1 expression. Hypoxia occurs when the oxygen tension is less than that required for cells to function normally in certain tissues. Tumor cells invade the surrounding tissue by forming invadopodia and degrading the surrounding basement membrane. The cell will make a small perforation where the invadopodia are formed, then elongate and mature, so that the cell can invade through the perforation into the surrounding tissue. In this process, Vimentin is needed. Therefore, the transcriptional regulation of the Vimentin protein is

one of the potential drivers of the emergence of EMT [22-24].

Eugenol is one of the main components that can be found in clove flowers (*Syzygium aromaticum*). Eugenol achieves a therapeutic effect by inhibiting the process of epithelial to mesenchymal transition (EMT) which can decrease the ability of cells to migrate. Eugenol has a strong hydrogen donor ability and is able to reduce the activity of damaging oxidants or as a free radical scavenger of superoxide, hydrogen peroxide, and other contaminants. Eugenol is also able to inhibit oxidative degradation such as lipid peroxidase [25].

Those things above were indicated by a decrease in the value of Vimentin expression in the treatment group (P+1 and P+2) with the administration of methanol extract of cloves (*Syzygium aromaticum*) 70 mg/kg BW orally. In comparison to the positive control group, vimentin expression significantly decreased in the treatment groups on days 18 and 36. In the treatment group, Vimentin staining was mostly detected in the apical cytoplasm of Sertoli cells. At P+1 with 18 days of treatment, the value of Vimentin expression was slightly smaller than P+2 with 36 days of treatment. The insignificant change in the interaction between the treatment groups and the length of treatment was indicated by a significant value of more than 0.05 ($p>0.05$) in the comparison between groups K+1 and K+2 and P+1 with P+2.Oral administration of cloves (*Syzygium aromaticum*) methanol extract at a dose of 70 mg/kg BW white rats (*Rattus norvegicus*) could reduce Vimentin expression in cryptorchid-induced Sertoli testicular cells for 18 and 36 days. However, the difference in the length of treatment days did not show any significant changes.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Author Contributions

Epy Muhammad Luqman (Supervision of MS research), Nisrina Dwi Andarheta (Writing of manuscript and submission), Nusdianto Triakoso (Experimental work for her MS degree), Wurlina Wurlina (Analysis), Nove Hidajati, (Interpretation of results), Juliano Mwenda Ntoruru (Antioxidant potential), Lita Rakhma Yustinasari (Surgeon).

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