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Chemical Castration Using Iron (III) Chloride Hexahydrate

(KEBIRI KIMIAWI MENGGUNAKAN FERIKLORIDA HEKSAHIDRAT)

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ABSTRACT

Chemical castration is a method that can be applied easily without any surgical intervention in animals. This study utilized iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) as a new material for chemical castration in mice. Twenty seven adult male mice were divided into five groups: $FeCl_3$ 20% (n = 6), $FeCl_3$ 10% (n = 6), $FeCl_3$ 5.0% (n = 6), $FeCl_3$ 2.5% (n = 6), and control NaCl 0.9% (n = 3). A 0.2 mL of NaCl 0.9% or $FeCl_3$ in various concentrations was injected intra-testicularly on each testis of the mice. Post-castration survival rate with LD_{50} values was obtained at the concentrations between 2.5-5.0% of $FeCl_3$ groups, and 100% mice survived in the control group. The size of testis and concentration of spermatozoa decreased, in contrast with the increased concentration of $FeCl_3$ solution used seven days post-injection compared to the control group.

Keywords**:** chemical castration; iron (III) chloride hexahydrate solution; survival rate; testis; mice

ABSTRAK

Kebiri/kastrasi kimiawi secara injeksi intra-testis merupakan metode pengebiriam yang dapat dilakukan dengan mudah tanpa prosedur bedah pada hewan. Penelitian ini memanfaatkan larutan besi (ferri/III) klorida ($FeCl_3$) sebagai bahan baru untuk tindakan kebiri kimiawi pada mencit. Mencit jantan dewasa umur lima bulan sebanyak 27 ekor dibagi dalam lima kelompok yaitu $FeCl_3$ 20% (n=6), $FeCl_3$ 10% (n=6), $FeCl_3$ 5,0% (n=6), $FeCl_3$ 2,5% (n=6) dan kontrol NaCl 0,9% (n=3). Larutan $FeCl_3$ sebanyak 0,2 mL diinjeksikan secara intra-testikel pada setiap organ testis. Daya hidup pascakebiri injeksi nilai LD 50 diperoleh pada kelompok $FeCl_3$ konsentrasi di antara 2,5-5,0 % dan kelompok kontrol 100 % hidup. Organ testis dalam skrotum mengalami pengecilan ukuran dan konsentrasi spermatozoa mengalami penurunan seiring dengan peningkatan konsentrasi larutan $FeCl_3$ yang digunakan setelah tujuh hari pasca injeksi dibandingkan dengan kontrol.

Kata-kata kunci: kastrasi per-injeksi; larutan besi (III) klorida heksahidrat; daya hidup; testis; mencit

INTRODUCTION

Castration is a medical procedure that performed to remove testis function in male animals that used for reproduction process. Castration is intended to control animal populations (McCarthy *et al.*, 2013) and livestock efficiency in productivity (Gonzalez *et al.*, 2010). The castration procedure is conducted through taking off the testis (Pineda and Dooley, 1984; Root Kustritz, 2012), ligation (Gonzalez *et al.*, 2010) and cutting (Pang *et al.*, 2009) ductus epididymis to stop semen (spermatozoa) distribution and circulatory system, hormonal vaccine (Ajadi and Oyeyemi, 2014) to induce immunological reactions, and chemical castration (Pineda and Dooley, 1984) to damage tissue by injection procedure.

The development of chemical castration had been conducted more than five decades using

various kinds of organics and inorganics material. Inorganic chemical of cadmium has been used as chemical castration in rabbits since five decades ago (Cameron, 1965). Further, calcium chloride dihydrate has been also tested as chemical castration material in goats (Jana *et al.*, 2005), dogs (Jana and Samanta, 2007), cat (Jana and Samanta, 2011) and cattle (Neto *et al.*, 2014). Moreover, zinc (Zn) has been tested in cats (Oliveira *et al.*, 2013) and its combination with gluconate also has been tested in dogs (Oliveira *et al.*, 2012). Commercial pharmaceutical products for chemical castration has been marketed as ZeuterinTM / EsterilSol™ (Ark Sciences, USA) and Neutresol® Injectable Solution (Abbott Laboratories, USA) with the active ingredient of zinc gluconate and arginine in aquabidest (Lau, 2012; Westlund, 2014). The commercial pharmaceutical products have been contraindicated in animals with cryptorchidism, scrotal dermatitis, testicular diseases and malformations, and also hypersensitivity. Thus, the development of a potential new raw material needs to be done without neglecting safety and effectiveness in animals.

Biodegradable metal materials e.g. iron, magnesium, calcium and zing have been introduced in medical applications (Hermawan, 2012a, 2012b; Yun *et al.*, 2009; Zheng *et al.*, 2014). However, until now, the use of a solution of ferrous metals as an active ingredient in chemical castration method of intra-testicular injection has not been reported. Thus, this research was aimed to use a solution of iron (III) chloride hexahydrate in different concentration as the active ingredient in the chemical castration of intra-testicular injection in mice. The survival rate, body weight, testicular morphology and spermatozoa viability post-chemical castration in mice were analyzed and reported.

RESEARCH METHODS

Solution Preparation for Chemical Castration

A stock solution of 20% $FeCl_3$ was made by dissolving 2 g of $FeCl_3.6H_2O$ powder (Nacalai Tesque, Japan) within 10 mL of aquabidest, which was further diluted into several concentrations of 10, 5, and 2.5% stock solution. A concentration of 5% NaCl was used as placebo.

Experimental Animals

Twenty seven adult mice (male, five months, DDY strain) were used in this study. All mice were acclimatized for 14 days prior to the chemical castration. All mice have received anthelmintic, antibiotic, and antifungal. Mice were divided into treatment groups $FeCl_3$ concentration of 20% (FeCl 20) (n = 6), 10% (FeCl 10) (n = 6), 5% (FeCl 5.0) (n = 6), 2.5% (FeCl 2.5) (n = 6) and 0.9% NaCl normal saline were used as controls (NaCl 0.9) (n = 3).

Intra-testicular Injection

Mice were handled and restrained comfortably in the supine position with the head position higher than the tail. Testicular fixation was conducted using the index finger to shifting the testis further into the scrotum. The chemical castration was conducted using a 1 mL syringe with a 27-gauge needle. A 0.2 mL of $FeCl_3$ or saline solution was injected and deposited into each testis at the middle part of the testis with the depth of ± 5 mm according to the treatment groups (see Fig. 1a).

Survival Rate of Mice after Intra-testicular Injection

The survival rate of mice was observed to assess acute mortality incidence (LD_{50}) at 3, 6, 12, and 24 hours post-chemical castration. The observation of mice mortality was then continued at 48, 72, 96, 120, 144, and 168 hours to assess long term of survival rate.

Body Weight

Absolute body weight was weighed using digital scales prior to castration and at seven days post-chemical castration. The percentage of body weight change was analyzed seven days post-chemical castration.

Testis Observation, Collection, and Weighing

Scrotal morphology inspection was conducted following by survival rate observation. Scrotal changes were recorded and described in results. Further, testis was collected at day seven post-castration through open surgical procedures. Mice were anesthetized using ketamine with a dose of 50 mg/kg of body weight combined with xylazine with a dose of 5 mg/kg body weight, intraperitoneally, prior to open castration.

The open castration was performed by cutting open the skin of the scrotum and tunica

vaginalis communis to reach testis after mice were fully anesthetized. Testis was separated from the scrotum by cutting the ligaments, fat, blood vessels, nerves and epididymal duct. The cauda epididymis was cut off and then stored in 10% of normal buffer formaldehyde (NBF) solution for further spermatozoa analysis. The preserved testicles in 10% NBF were then digitally documented among treatment groups.

The absolute weights of testis were weighed using a digital scale. The absolute weight of testis was calculated by comparing the absolute weight of the testis to the body weight of mice. The testis diameter in length and wide size were then measured using a caliper in millimeter scale. The relative weights of testis were calculated by comparing the absolute testis to body weight in units of percent.

Spermatozoa

Surgical open castration procedure was used to collect testis organ as mentioned in previous subchapter. Semen was collected from cauda epididymis of exposed testis for assessment of morphology and concentration of spermatozoa. Cauda epididymis was chopped using scissor. Afterward, the chopped tissue was then added by 50 uL of eosin negrosin solution in Eppendorf tube and its vortexes until well mixed for morphological assessment. A 10 uL of the mixture was then dropped onto objective glass, smeared thinly, and dried. The existence of spermatozoa in the semen was observed using a light microscope from the smear. The used mixture (50uL) was then added 950 uL of formol-saline and its vortexes again until well mixed for spermatozoa counting. Spermatozoa concentration was then calculated by using Neubauer hemocytometer counting chamber under a light microscope.

Data Analysis

Data were analyzed to determine the differences between chemical castration groups. The data were then analyzed by using SPSS v.16.0 software (SPSS Inc., USA) with a further test on a one-way ANOVA post hoc Duncan test with 95% confidence interval. P values of <0.05 indicate significant differences between the groups.

RESULTS

Chemical castration could be done easily without open surgical procedure, in which the active substance was injected directly into the testis (Fig. 1a). Mice which were injected showed a slight decrease activity up to without showed any movements in a few minutes after chemical castration. Mice then started activities within 30-60 minutes further. The LD_{50} value showed the mortality rate of FeCl 20 and FeCl 10 groups were more than 50% of all population of mice within 24 hours. Effective dose values were shown in the range concentration of $FeCl_3$ solution between 2.5% (83%) to 5.0% (33%) (see Fig. 1b and Table I). Body weight of mice decreased after seven days post-injection with the percentage changes were not significantly different between groups ($P>0.05$) (see Table 1).

The survival rate of mice up to 7 days post-chemical castration was shown at NaCl 0.9, FeCl 2.5 and FeCl 5.0 groups. Scrotum appearance differed between the survive treatment groups 7 days post-chemical castration. The FeCl 5.0 group had the smallest size compared to other groups (see Fig. 1c-e). The scrotal pouch of FeCl 5.0 group underwent shrinkage due to testis was contracted into the abdominal cavity (see Fig. 1e). However, in the FeCl 2.5 and control group, the scrotum pouch was appearing externally and testis still filling the scrotum pouch (see Fig. 1c and Fig. 1d).

The size of testis was seen decreased with the increasing concentration of $FeCl_3$ solution has the following trend of NaCl 0.9> FeCl 2.5 > FeCl 5.0 (Fig. 1f-g and Table I). Absolute and relative organ weight of testis showed a decrease in line with testis size reduction. The FeCl 5.0 group has the lower absolute and relative weights when compared with FeCl 2.5 and control. The wide and average diameter of testis from the measurement showed significant differences between the groups ($P<0.05$) (see Table I).

The assessment of the presence of spermatozoa through the smeared eosin negrosin of semen also showed in similar results. The FeCl 5.0 group showed that the presence of spermatozoa in fewest amounts compared to the NaCl 0.9 and FeCl 2.5 groups (see Fig. 1i-k).

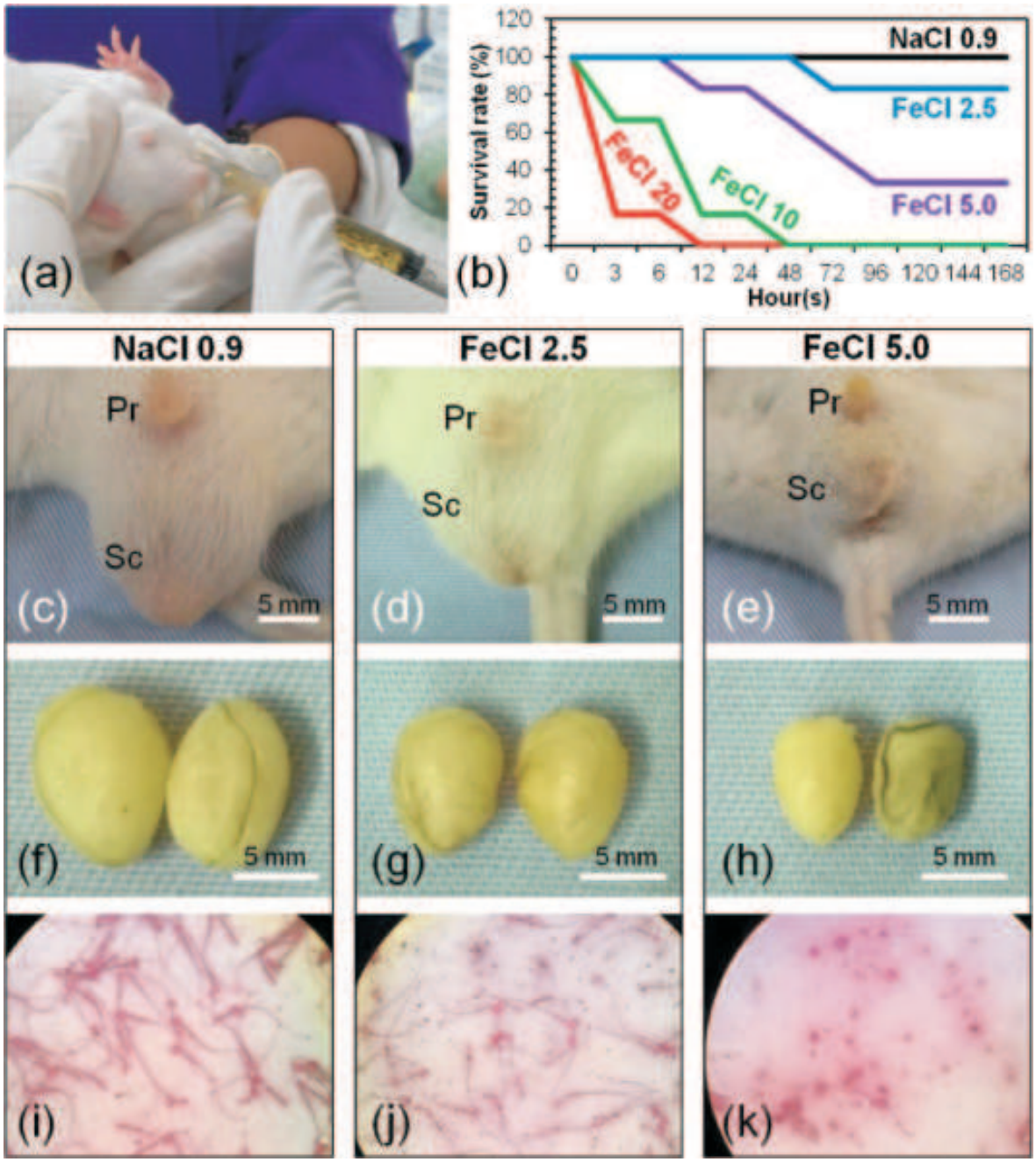

Figure 1. Chemical castration using several concentrations of iron (III) chloride hexahydrate solution and biological performance of mice after seven days observation. (a) Chemical castration procedure, (b) survival rate, (c-e) scrotum appearance, (f-h) testis (preserved in 10% normal buffer formaldehyde), (i-k) spermatozoa (in eosin negrosin staining, 400x of magnification). Note: Pr = prepuce, Sc = scrotum.

Further analysis of the concentration of spermatozoa was indicated that FeCl 5.0 group had the lowest sperm concentration compared to FeCl 2.5 and control groups ($P<0.05$) (Table I).

DISCUSSIONS

Chemical castration technique can be conducted quickly and easily when compared to open surgical castration or ligation procedures.

Table 1. The biological profile of mice after seven days post-chemical castration with a concentration of 0.9% NaCl (NaCl 0.9), 2.5% $FeCl_3$ (FeCl 2.5), and 5.0% $FeCl_3$ (FeCl 5.0).

Parameters	Treatments			
	NaCl 0.9	FeCl 2.5	FeCl 5.0	P-value
Survival rate (%)	100.0±0.0	83.3±0.0	33.3±0.0	-
Body weight				
Absolute (g)	39.67±5.51[a]	36.40±2.97[a]	33.00±2.83[a]	0.230
Changes (%)	1.33±1.53[a]	-3.00±3.39[a]	-5.00±5.66[a]	0.163
Testis morphology				
Absolute weight (g)	0.12±0.04[b]	0.10±0.02[ab]	0.07±0.01[a]	0.134
Relative weight (% body weight)	0.30±0.05[b]	0.27±0.04[ab]	0.20±0.06[a]	0.138
Diameter of lenght (mm)	0.84±0.08[b]	0.73±0.05[ab]	0.70±0.06[a]	0.060
Diameter of wide (mm)	0.59±0.08[b]	0.53±0.03[ab]	0.45±0.03[a]	0.021
Mean diameter (mm)	0.71±0.07[b]	0.63±0.02[ab]	0.58±0.05[a]	0.024
Spermatozoa (10^9 sel.mL^{-1})	2.21±0.10[b]	1.58±0.18[b]	0.12±0.07[a]	0.004

Note: Data were shown as mean with standard deviation (x±SD). The same letter in a row shows the differences was not significant (P>0.05)

Chemical castration is considered as an option in an animal to control the population with lower costs when compared to open surgical castration (Jana and Samanta, 2011). The $FeCl_3$ solution with gradual level concentrations of 20%, 10%, 5%, and 2.5% as an active agent for chemical castration in this study was also showed a similar to others. The testis profile was decreased in some biological parameters along with increased concentrations of $FeCl_3$ solution (Fig. 1 and Table I). Chemicals agents such as lactic acid (Nishimura *et al.*, 1992), calcium chloride (Jana and Samanta, 2007), and zinc gluconate (Soto *et al.*, 2007) were able to reduce the dimensions of testicular organs, increasing the number of spermatozoa abnormalities and decrease the concentration of spermatozoa along with increasing concentration.

The $FeCl_3$ solution is a chemical that commonly used to induce thrombosis in several animals for cardiovascular diseases model (Li *et al.*, 2013). The $FeCl_3$ solution is acidic, highly corrosive, toxic, and could work as agents that cause strong dehydration. The potential chemical properties of $FeCl_3$ can be used as an active agent for chemical castration by adjusting the solution concentration in order to obtain the appropriate dose and safety in the application.

Chemical castration using calcium chloride solution with the addition of lidocaine could cause temporary discomfort and last in about five minutes min in cats (Jana and Samanta, 2011). In mice, the injection of the $FeCl_3$ solution also showed the similar evidence with a longer duration because the volume ratio of the solution with testicular volume was greater compared to that application in the cat. Mice received a dose of 0.2 mL/testis when compared to cat applications that use of 0.25 mL/testis size with greater testis volume.

A gradual concentration is generally used to determine the dose of death or lethal doses (LD) 50 in the development of a pharmaceutical preparation. High mortality in this study occurred within 24 h post-injection in groups of FeCl 10 and FeCl 20 (Fig. 1b and Table I). The chemical castration using $FeCl_3$ solution in testis will be distributed systemically in the body through the blood vessels of mice. Injection $FeCl_3$ solution with a concentration of 10% inside in the blood vessels was able to induce endothelial cell damage, platelet aggregation, and thrombus formation quickly (acute) in mice (Tseng *et al.*, 2006). The $FeCl_3$ solution in higher concentrations of up to 20% was reported to be the most effective in thrombus formation in blood vessels of mice (Surin *et al.*, 2010). Thrombus is a blockage that occurs in the circulatory system as a result of the aggregation of blood cells to be clumps and interferes with blood perfusion in tissue (Barr *et al.*, 2013). Large thrombus formed quickly that causing general thrombosis and then it cause disruptions of general circulation in the body. General acute hypoxia occurs due

to disruption in the distribution of oxygen by red blood cells into tissues, and it causing tissue death within a short time.

The chemical castration of $FeCl_3$ solution in a lower concentration between 2.5-5.0% had a lower mortality rate compared to the concentration of 10-20%, where mice could survive up to seven days post-chemical castration (Fig. 1b and Table 1). General thromboses were not clinically visible on the injection of the $FeCl_3$ solution with a concentration range of 2.5-5% in this study. Injection $FeCl_3$ solution in testis with a concentration between 2.5-5% also caused a thrombus, however, the thrombus effect on the circulating blood and the vascular was not seen in clearly (Li *et al.*, 2013). In detail, a solution of $FeCl_3$ with a concentration of 2.5% was also able to induce thrombus with a very small size and not enough to interrupt the circulation of blood perfusion. While the concentration of 5% $FeCl_3$ solution in one minute was able to induce greater thrombus compared to the concentration of 2.5%, however, the tissue effects also was not visible.

The chemical castration using $FeCl_3$ solution at FeCl 2.5 and FeCl 5.0 groups in this study might lead to local thrombosis to the testis (Fig. 1b and Table 1). Testis has a blood-testis barrier (BTB) system in the seminiferous tubules which play an important role to maintain homeostasis in equilibrium state (Chihara *et al.*, 2013). The BTB capillaries also play a crucial role in maintaining the process of spermatogenesis (Cheng and Mruk, 2012). The local thrombosis occurred on the BTB capillary of testis post-injection of $FeCl_3$ solution. Local thrombosis in the circulatory system of BTB capillaries can cause organ infarction and develop into ischemia. Ischemia is an oxygen deficiency in the tissue due to interruption of oxygen distribution caused by a narrowing or blockage of blood vessels (Oštádal *et al.*, 1999). Testicular ischemia disrupts the distribution process of nutrients and a metabolic waste of cells, thus, the process of spermatogenesis was disrupted. The incidence of ischemia at chemical castration using $FeCl_3$ solution was similar to the incidence of testicular torsion on the testis (Özokutan *et al.*, 2000). Testicular torsion could lead to cells apoptosis, cells atrophy and a decrease of spermatogenesis process that mediated by proinflammatory cytokines such as TNF-α, IL1-α, and IL1-α. The TNF-α cytokines produced by round spermatid, pachytene spermatocytes, and macrophages of the testis. While cytokines IL1-α and IL1-α was produced by the Sertoli cells, macrophages of the testis, and post-meiotic stem cells (Lysiak, 2004).

Chemical castration of FeCl 5.0 and FeCl 2.5 groups also caused shrinkage of the size of scrotum and testis morphology compared NaCl 0.9 group (Fig. 1c-h and Table 1). The shrinkage of testis was caused by local thrombosis and disturbance of the circulatory system. The testis function to produce spermatozoa was declining due to the shrinkage of the size. Spermatozoa formed through spermatogenesis process that started from spermatogonia cells into spermatocytes and becomes spermatids that occur in the seminiferous tubules of testis (Hafez and Hafez, 2000). This process played the main role to maintain the presence of spermatozoa for reproduction purposes (Yoshida *et al.*, 2006). The process of spermatogenesis in mice occured with one spermatogenic wave that took a period of 8.6 days (Davis, 2012). Spermatogenic waves usually occur in overlapping one to other waves and varied age spermatozoa would appear on it (Evans *et al.*, 2014). The concentration of spermatozoa showed that the FeCl 2.5 and FeCl 5.0 groups have a lower (in less amount) concentration compared to NaCl 0.9 control group ($P<0.05$) (Fig. 1i-k and Table 1). The concentration of spermatozoa in the semen shows in less or zero known as oligospermia or azoospermia, respectively, that happened after the chemical castration in dogs (Jana and Samanta, 2007; Pineda and Dooley, 1984) and cat (Jana and Samanta, 2011).

Chemical castration using a solution of calcium chloride ($CaCl_2$) in dogs with high concentrations (10-20%) was reported not effective, where the testis still produced sperm (Leoci *et al.*, 2014). On their study, the effectiveness of $CaCl_2$ solution for chemical castration were shown at a concentration of 30% after one year of observation. The use of zinc gluconate solution with a concentration of 1.3% was also effective as chemical castration in dogs and therein no semen ejaculated after 30 days post-injection (Tepsumethanon *et al.*, 2005). This study, chemical castration using $FeCl_3$ solution with a low concentration of 2.5-5.0% in the short term for seven days post-injection might cause permanent damage to the testis. It can be seen that spermatozoa production, on semen smear analysis, became lower than the control (see Fig. 1 and Table 1). It appears that the testis failure in producing spermatozoa due to $FeCl_3$ solution injection. Although the effective dose was not obtained in this study, however, some biological profile parameters on the observation of testis

showed in lower values compared to the control group ($P<0.05$).

Commercial products of chemical castration had a mode of action by stimulating the local inflammation in the testis. The inflammation process then impaired the process of spermatogenesis (Westlund, 2014). The testis of cat underwent coagulation necrosis, fibrosis, and then developed into hyalinization after chemical castration (Jana and Samanta, 2011). In addition, tissue damage inducing the release of chemotactic factors to activate of body immunological response against foreign substances that exist in the tissue (Heath and Arowolo, 1987). In contrast to the mode action of commercial products of chemical castration, our study using $FeCl_3$ solution with low concentration (2.5-5.0%) were able to induces thrombosis through local hypertonic action and iron (III) hexahydrate was able to kill the testis cells locally. The hypertonic solution could cause damage to walls of cell structure in which the intracellular fluid was drawn out into extracellular parts thus the cells underwent rapid shrinkage (Pascual *et al.*, 2003). Toxicant in solution was able to impair and damage of cellular function when it existed in the cell environment (Mahmoudi *et al.*, 2009). Local thrombosis that occured by obstructing the process of distribution and disposing of cell metabolism that leads to organ damage. The damages that occured at the cellular level would impact on the tissue level thus the function of organs were disturbed, decreased in its function, and this lead to a total tissue damage which level depended on the potential of the material deposited in the tissues.

CONCLUSIONS

This study has successfully demonstrated the potential of $FeCl_3$ solution to reduce the function of the testis within seven days after intra-testicular injection and potentially could be developed as a new active substance in chemical castration.

SUGGESTION

Further development is needed to determine the long-term effect and obtain the best of effective dose in reducing the function of testis.

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