Testes biopsy analysis and hormonal reproduction levels of pig tail macaque *(Macaca nemestrina)* injected with testosterone enanthate (TE) and depot medroxy progesterone acetate (DMPA)

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Abstrak

Tujuan penelitian ini adalah untuk menganalisa testis hasil biopsi dan mengetahui kadar hormon reproduksi pada beruk (Macaca nemestrina) jantan yang disuntik kombinasi testosteron enantat (TE) dan depot medroksiprogesteron asetat (DMPA). Sebanyak 6 beruk jantan berumur antara 6 tahun sampai 8 tahun digunakan sebagai sampel. Masa adaptasi 3 bulan, kemudian dilakukan penyuntikan TE dengan dosis 32 mg setiap minggu selama 6 minggu dan dilanjutkan setiap 3 minggu sampai minggu ke 24, sedangkan penyuntikan DMPA dilakukan tiap 6 minggu dengan dosis 40 mg sampai minggu ke 18. Selain itu dilakukan pula pengukuran volume testis tiap 3 minggu dan pengambilan darah setiap 6 minggu untuk melihat kadar hormon gonadotropin dan hormon steroid. Biopsi testis dilakukan pada minggu ke-30 dan minggu ke-48. Pembuatan sajian histologi testis dengan metode parafin dan pewarnaan hematoksilin-eosin (HE). Hasil penelitian menunjukkan bahwa rata-rata volume testis kanan dan kiri mengalami penurunan pada masa perlakuan yaitu dari 18.35 cm3 ± 9.35 dan 19.02 cm3 ± 10.88 (minggu ke 0) menjadi 6.70 cm3 ± 3.80 dan 7.02 cm3 ± 4.61 volume terendah pada minggu ke 21. Kemudian pada masa pemulihan akan meningkat kembali yaitu 20.34 cm3 ± 7.87 dan 21.75 cm3 ± 7.09 pada minggu ke 48. Rata-rata diameter tubulus seminiferus dan skor spermatogenesis testis kanan dan kiri pada akhir pengaruh TE dan DMPA (minggu ke 30) adalah 0.13 mm ± 0.027 dan 0.13 mm ± 0.026 serta skor 5.08 ± 2.67 dan 5.41 ± 2.51. Pada akhir penelitian (masa pemulihan, minggu ke 48) diameter tubulus menjadi 0.18 mm ± 0.029 dan 0.18 mm ± 0.026 serta skor spermatogenesis 7.51 ± 2.14 dan 7.57 ± 1.59. Di samping itu tubulus seminiferus pada minggu ke 30 mengalami hialinisasi dan fibrosis. Kadar hormon testosteron total, testosteron bebas, dan estrogen umumnya meningkat tajam sampai minggu ke 6 dan akan menurun kembali tetapi masih lebih tinggi dibandingkan dengan kadar basalnya sampai minggu ke 30. Pada masa pemulihan kadar estradiol meningkat tajam kembali sampai akhir penelitian. Kadar hormon FSH dan LH menurun sampai minggu ke-6, kemudian hormon FSH akan meningkat kembali sampai akhir penelitian, sedangkan pada kadar hormon LH penurunan tersebut tetap dipertahankan sampai akhir penelitian. Dari hasil penelitian disimpulkan bahwa penyuntikan kombinasi TE dan DMPA akan mempengaruhi struktur histologi testis yang ditandai dengan pengecilan diameter tubulus seminiferus, hialinisasi, dan fibrosis serta menghambat perkembangan spermatogenesis. Gangguan yang terjadi pada testis tersebut lebih disebabkan oleh adanya hambatan terhadap umpan balik pada poros hipotalamus-hipofisis-testis. ***(Med J Indones 2001; 10: 137-43)***

Abstract

The objective of this study is to analyse the testes biopsy and to know the hormone level reproduction of pig tail macaque (Macaca nemestrina) injected with testosterone enanthate (TE) and depot medroxyprogesterone acetate (DMPA). Six pig tail male macaques, age 6 - 8 years old were used as sample. Three months after adaptation period, each animal was injected intra muscularly with 32 mg TE each week starting at week zero up to the sixth week. The treatment was continued every 3 weeks after the sixth week up to the 24th week. 40 mg of DMPA was injected intramuscularly at week zero, and continued every week up to week 18. Volume of the testes was taken every three weeks and blood samples for examination of gonadotropin hormone and steroid hormone were taken at 6 week intervals. Testes biopsy was performed at week 30 and week 48. Preparation of testes histological slides were made using the paraffin method and stained with hematoxylin-Eosin (HE). The results of this study showed that both testes volume decreased i.e. 18.35 cm^3 ± 9.35 and 19.02 cm^3 ± 10.88 (at week zero) to 6.70 cm^3 ± 3.80 and 7.02 cm^3 ± 4.61 (lowest volume at week 21). In recovery period, the testes volume increased to 20.34 cm^3 ± 7.87 and 21.75 cm^3 ± 7.09. The diameter of the seminiferous tubules and the score of spermatogenesis (righ and left testes) at week 30 were 0.13 mm ± 0.027 and 0.13 mm ± 0.026 and score were 5.08 ± 2.67 and 5.41 ± 2.51. At week 48, both diameter of seminiferous tubules and spermatogenesis score increased to become 0.18 mm ± 0.029 and 0.18 mm ± 0.026, and score were 7.51 ± 2.14 and 7.57 ± 1.59. During this period, hyalinization and fibrosis of seminiferous tubule occurred. By week 6, the total testosterone, free testosterone, and estrogen hormone levels increased quite sharply and then decreased but still higher than base levels of hormone. In the recovery period, estrogen hormone increased significantly until the end of observation (week 48). FSH and LH hormone levels decreased until week 6, then the FSH hormone levels increased until the end of observation, while the LH hormone level is still lower than base level. Conclusion of this study is the injection of TE and DMPA combination will alter the histological structure of the pig tailed

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macaque testes i.e. decreasing the diameter of the seminiferous tubules, suppressing spermatogenesis and hyalinisation and fibrosis of seminiferous tubules. The damages of this structure are likely caused by inhibition of feedback mechanism of hypothalamus-hypophysis-testes. ***(Med J Indones 2001; 10: 137-43)***

Keywords: *Macaca nemestrina, seminiferous tubule, spermatogenesis, gonadotropin hormone, steroid hormone.*

The aim of male hormonal contraception is to suppress spermatogenesis process via the hypothalamus-hypophysis- testes axis.[1-2] This method caused a decrease in gonadotropin production, and influenced the spermatozoa production and quality. The administration of high doses of testosteron enanthate (TE) to healthy men through intra muscular injection induced azoospermia and is followed by lower spermatozoa quality.[3] Data from 10 study centers in 7 cities concluded that administration of 200 mg/week TE to 271 healthy and fertile men caused azoospermia in 157 (65%) of the volunteers after up to six months of treatment.[4]

Combination of TE and DMPA has already been investigated several times for effectiveness. Knuth *et al* conducted a trial on 12 volunteers who was injected with 200 mg 19-nortestosterone hexyloxyphenyl propionate (19NT-HPP) and 250 mg DMPA, azoospermia were achieved in 6 men, while 4 men achieved oligozoospermia with concentrations < 2 x 10^6/mL, and the remaining 2 men produced only a single sperm per ejaculate.[5] In contrast, a previous study in Indonesian men given combination of TE and DMPA injection demonstrated higher rates of azoospermia. In a trial conducted by Pangkahila on 10 volunteers, azoospermia were achieved in all volunteers (100%),[6] and one conducted by Moeloek on 45 volunteers, azoospermia was achieved in 95.7% of the volunteers.[7]

The studies above showed that combination of TE and DMPA induced azoospermia. However, the exact effect on spermatogenesis in the testes is still unclear. Therefore, it is very important to understand the inhibition of spermatogenesis by injection of TE and DMPA combination. Observations using testes biopsy from men are considered unethical so therefore, it is important to carry out the investigation using animals who evolutionally are close with humans. The aim of this investigation is to perform an analysis of testes biopsy and hormonal reproduction of pig tail macaque testes injected with combination TE and DMPA.

METHODS

Animal and treatment

Six male pig tail macaques (Macaca nemestrina), age 6-8 years old and 6-12 kg body weight were used in this investigation. They were obtained from the Primate Research Center of Bogor Agriculture Institute, West Java, Indonesia. Animals were housed in cages measuring 23x21x12 inches, maintained at a room temperature of 20°-29°C, and well ventilated. The standard monkey-chow was imported from Seafoods Enterprise Co. Ltd. Thailand. The investigation was divided into three periods : adaptation period for 3 months from –12 week to zero week; treatment period for 6 months from zero week to 24th week; recovery period for 6 months from week 24 to week 48. Three months after the adaptation period, each animal was injected intra muscularly with 32 mg TE each week starting at week zero up to the sixth week. The treatment was continued every 3 weeks after the 6th week up to the 24th week. Fourty mg DMPA was injected intramuscularly at week zero, and continued every week up to week 18.

Testicular volume and testicular biopsy

The testicular volume measurements were taken every three weeks. The measurement was conducted using the Bercovitch & Rodriguez method: $TV = (\eta W^2L)/6$; TV = testes volume, W = width of the testes, L = length of the testes.[8] A surgical biopsy was carried out at week 30 and week 48. Standard biopsy specimens (3x3x3 mm3) were taken under anesthesia. The biopsy specimens were fixed in Bouin Solution, and processed using the parafin method; 5 μm thick sections were stained with hematoxylin and eosin. Light microscopy and microprojector examination were performed. To assess the spermatogenesis development in the seminiferous tubule, the scoring method from Johnsen was used.[9]

Hormone measurements

Blood samples for hormone measurements were obtained from the femoral vein. Serum was frozen at –20°C until assayed. Serum steroids (total testosterone, free testosterone, and estradiol) were measured using RIA kits using the coat-A-Count ^{125}I technique as described by the producer (Diagnostic Products Corporation, Los Angeles, CA 90045). FSH and LH were measured using double antibody RIA kits and the technique described by the producer (Diagnostic Products Corporation, Los Angeles, CA 90045).

RESULTS

Body weight and testes volume

The body weight of each pig tail macaque at the week 0 before injection averaged 9.10 kg ± 3.28. During treatment, their body weight increased. By the end of week 21, the average weight rate reached up to 11.00 ± 2.87. At the recovery period i.e. between week 30 to week 48, the body weight was stable around 9.82 kg to 11.02 kg, as shown in Table 1.

The average volume for both right and left testes at the week 0 were 18.35 cm^3 ± 9.35 and 19.02 cm^3 ± 10.88 respectively. During the treatment testes volume decreased and the lowest was at the week 21; the average volume of right and left testes were 6.70 cm^3 ± 3.80 and 7.02 cm^3 ± 4.61 respectively. During the recovery period, each testes volume increased and by week 48, their volumes were larger compared to the volume at week 0, i.e. 20.34 cm^3 ± 7.87 (right testes) and 21.75 cm^3 ± 7.09 (left testes). See Table 1.

Diameter of seminiferous tubules and score of spermatogenesis

After injection with TE and DMPA at week 30, the average diameter of tubulus seminiferous were 0.13 mm ± 0.027 (right testes) and 0.13 mm ± 0.026 (left testes). During the recovery period, their diameters increased (at week 48) to 0.18 mm ± 0.029 (right testes) and 0.18 mm ± 0.026 (left testes). See Table 2.

Table 1. Body weight and right and left testes volume before, during, and after injection with combination TE and DMPA

Weeks	Body weight (kg) ± SD	Testes volume (cm^3) ± SD	
		Right	Left
-12	8.28 ± 3.07	18.48 ± 9.33	18.25 ± 8.35
0	9.10 ± 3.28	18.35 ± 9.35	19.02 ± 10.88
3	9.48 ± 3.35	14.05 ± 9.31	14.64 ± 6.94
6	10.47 ± 3.64	13.64 ± 7.00	13.64 ± 9.25
9	10.25 ± 3.24	9.42 ± 3.89	10.01 ± 6.39
12	10.38 ± 3.21	8.22 ± 3.65	8.61 ± 4.50
15	10.67 ± 3.17	9.63 ± 5.17	12.94 ± 8.89
18	10.81 ± 3.04	11.65 ± 4.26	9.07 ± 5.62
21	11.00 ± 2.87	6.70 ± 3.80	7.02 ± 4.61
24	10.95 ± 2.71	8.07 ± 4.08	8.92 ± 5.78
27	10.73 ± 2.82	8.39 ± 6.22	8.07 ± 4.82
30	10.58 ± 3.13	8.72 ± 3.95	10.31 ± 4.52
33	10.53 ± 2.58	12.98 ± 9.34	13.40 ± 0.87
36	10.62 ± 1.42	11.93 ± 8.59	13.21 ± 0.48
39	10.70 ± 3.14	17.95 ± 9.20	17.75 ± 2.41
42	11.02 ± 2.87	17.05 ± 9.20	18.80 ± 9.93
45	9.82 ± 3.19	16.47 ± 6.38	17.68 ± 6.52
48	9.82 ± 3.35	20.40 ± 7.87	21.75 ± 7.09

Table 2. Diameter of the seminiferous tubules and scores of the testes spermatogenesis before, during, and after injection with combination TE and DMPA

Animal no	week-30			
	Diameter of seminiferous tubules (mm) ± SD		Scores of spermatogenesis ± SD	
	Right	Left	Right	Left
1	0.10 ± 0.026	0.10 ± 0.025	1.66 ± 1.21	2.50 ± 2.49
2	0.14 ± 0.019	0.14 ± 0.024	6.08 ± 1.63	6.62 ± 1.66
3	0.14 ± 0.023	0.14 ± 0.017	7.20 ± 1.55	7.24 ± 1.64
4	0.08 ± 0.015	0.08 ± 0.012	1.18 ± 0.27	1.41 ± 0.66
5	0.16 ± 0.015	0.15 ± 0.017	6.62 ± 1.99	6.62 ± 1.63
6	0.14 ± 0.023	0.14 ± 0.020	7.86 ± 0.72	8.06 ± 0.88
Σ	0.76	0.75	30.50	32.45
X ± SD	0.13 ± 0.027	0.13 ± 2.67	5.08 ± 2.67	5.41 ± 2.51
Animal no	week-48			
	Diameter of seminiferous tubules (mm) ± SD		Scores of spermatogenesis ± SD	
	Right	Left	Right	Left
1	0.12 ± 0.031	0.13 ± 0.037	3.20 ± 2.37	4.47 ± 3.06
2	0.18 ± 0.029	0.18 ± 0.027	8.86 ± 1.04	8.82 ± 1.01
3	0.19 ± 0.025	0.18 ± 0.017	8.90 ± 1.17	8.64 ± 1.64
4	0.20 ± 0.037	0.16 ± 0.025	6.36 ± 3.75	6.02 ± 3.98
5	0.19 ± 0.027	0.20 ± 0.023	9.06 ± 1.12	8.70 ± 1.39
6	0.21 ± 0.023	0.21 ± 0.028	7.86 ± 0.73	8.50 ± 0.96
Σ	1.09	1.06	45.06	45.42
X ± SD	0.18 ± 0.029	0.18 ± 0.026	7.51 ± 2.14	7.57 ± 1.59

The average score of spermatogenesis in the seminiferous tubule epithelium at week 30 were 5.08 ± 2.67 (right testes) and 5.41 ± 2.51 (left testes). These scores indicated that spermatogenic cells in seminiferous tubule do not contain spermatozoa and spermatid cells were very few or even not available. Two of pig tail macaques were in deep hyalinization (see Figure 1). During the recovery period, histological structure of seminiferous tubule epithelium improved, and these are shown through increasing scores at week 48, i.e. 7.51 ± 2.14 (right testes) and 7.57 ± 1.59 (left testes). See Table 2.

Steroid hormones

Levels of total testosterone hormone, free testosterone, and estradiol at week 0 were 1.98 ng/mL ± 1.0, 2.69 pg/mL ± 1.54, and 6.29 pg/mL ± 7.48 respectively. For 6 weeks after the first injection, the levels increased quite sharply, i.e. 8.52 ng/mL ± 1.52, 17.4 pg/mL ± 4.41, and 20.79 ng/mL ± 20.78 respectively. After week 6 the average level of the three steroid hormones decreased. Total testosterone levels by week 30 almost reached their basal levels (week 0), while free testosterone by week 30 were quite high (4.54 pg/mL ± 0.66) compared to week 0, and they came to their basal levels during the recovery period at week 36. Estradiol hormone levels during the treatment decreased to the lowest level by week 24 (2.43 pg/mL ± 4.81) and from week 30 to week 48 they increased drastically (10.18 pg/mL ± 6.04 to 18.74 pg/mL ± 4.71). See Table 3.

Table 3. Concentration of the steroid hormones serum before, during, and after TE and DMPA injection

Weeks	Steroid hormones (n = 6)		
	Total testosterone (ng/mL) ± SD	Free testosterone (pg/mL) ± SD	Estradiol (pg/mL) ± SD
-12	3.08 ± 2.78	4.55 ± 3.26	2.45 ± 1.09
0	1.98 ± 1.00	2.69 ± 1.54	6.29 ± 7.48
6	8.52 ± 1.52	17.40 ± 4.41	20.79 ± 20.78
12	5.55 ± 3.24	4.28 ± 0.94	9.89 ± 9.88
18	3.58 ± 2.27	5.09 ± 1.59	4.65 ± 3.32
24	4.31 ± 2.15	10.75 ± 4.42	2.43 ± 4.81
30	2.96 ± 2.18	4.54 ± 0.66	10.18 ± 6.04
36	3.12 ± 4.47	1.27 ± 0.32	16.68 ± 10.35
42	3.24 ± 3.76	3.43 ± 2.67	22.76 ± 3.49
48	5.09 ± 2.97	11.50 ± 2.66	18.74 ± 4.71

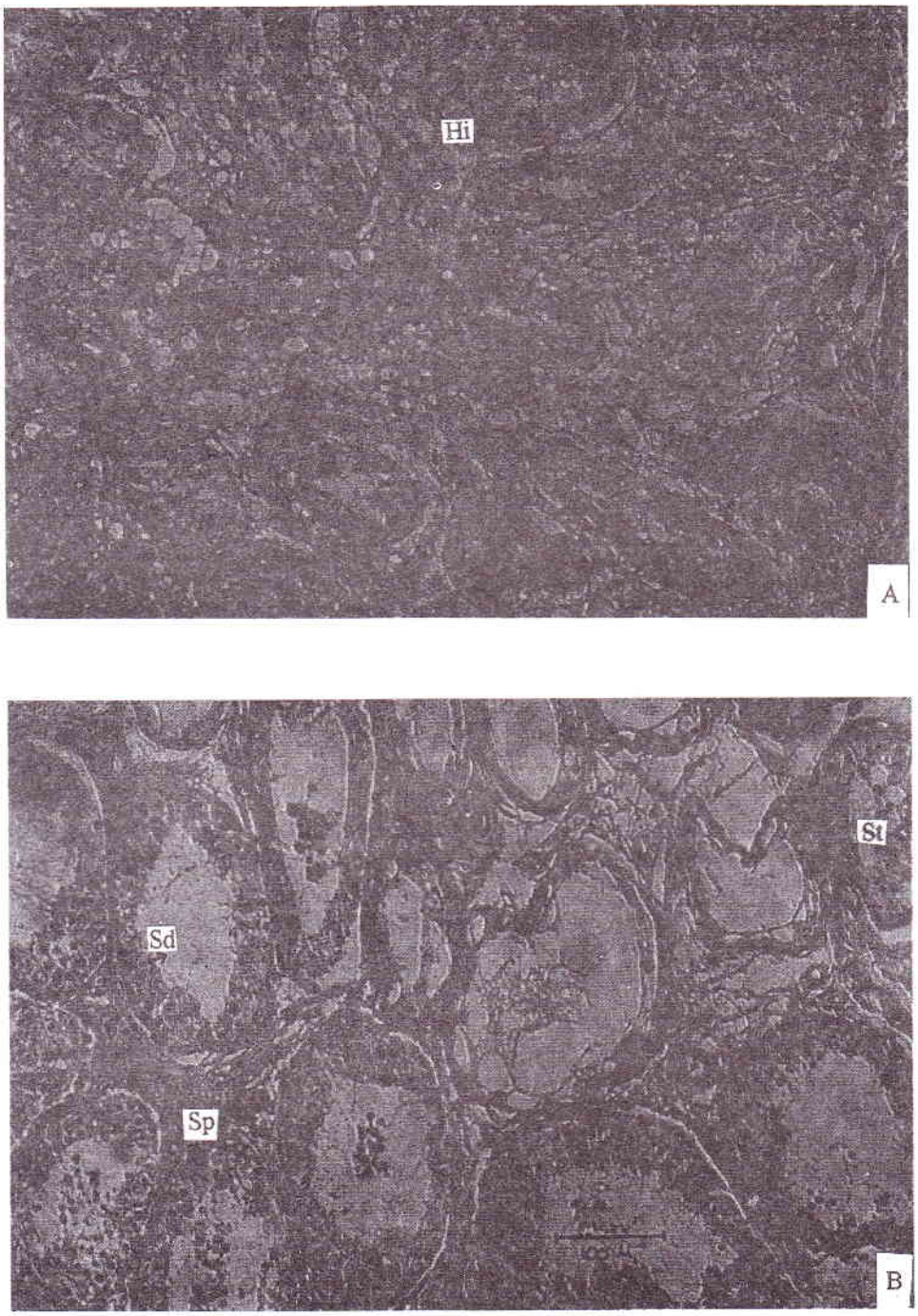

Figure 1. Histological structure of the testes. A: Structure of the testes at week-30; B: Structure of the testes at week-48. Sp : Spermatogonia; St : Spermatocyte; Sd : spermatid; Hi : Hyalinization.

Gonadotropin hormones

The average concentration of gonadotropin hormones FSH and LH at week 0 were 2.44 mIU/mL ± 1.33 and 0.10 mIU/mL ± 1.33 and 0.10 mIU/mL ± 0.00 respectively. After injections of TE and DMPA, FSH level decreased until week 12 (28 mIU/mL ± 0.18). After week 12 it increased gradually and reached the highest level by the week 36 (4.91 mIU ± 6.48). After given the first injection and until the study completed. LH hormone, however, decreased and reached the lowest level at the week 6, 30, and 48 i.e. 0.00 mIU/mL, 0.04 mIU/mL ± 0.07, and 0.01 mIU/mL ± 0.01 respectively (see Table 4).

Table 4. Concentration of the FSH and LH hormones before, during, and after TE and DMPA injection

Weeks	Gonadotropin hormones (n = 6)	
	FSH (mIU/mL) ± SD	LH (mIU/mL) ± SD
-12	3.06 ± 1.06	0.12 ± 0.06
0	2.44 ± 1.33	0.10 ± 0.00
6	0.26 ± 0.13	0.00 ± 0.00
12	0.28 ± 0.18	0.07 ± 0.10
18	2.26 ± 2.72	0.09 ± 0.12
24	2.41 ± 2.18	0.05 ± 0.09
30	4.01 ± 4.67	0.04 ± 0.07
36	4.91 ± 6.48	0.00 ± 0.00
42	2.99 ± 4.33	0.01 ± 0.03
48	1.11 ± 2.16	0.01 ± 0.01

DISCUSSION

Results of the study on body weight of pig tail macaques indicate that the feedback system disorder of hypothalamus-hypophysis-testes could not be demonstrated by only measuring their body weight. Suhana et al.(1999) reported that the two groups of cynomolgus macaques *(Macaca fascicularis)* that were injected with combination of TE and DMPA and were fed with different concentrations of fat and protein resulted in different body weights. This difference, however, has no effect on the feedback mechanism of both groups.[10]

Results indicate that there is a relationship between injection of TE and DMPA and testes volume. Study on measurement of seminiferous tubules diameter and scores of spermatogenesis shows that decrease of testes volume during injections of TE and DMPA have positive correlation with the changes in histological structure of testes. Decrease of testes volume is in line with diameter of seminiferous tubules which becomes smaller and also with the scores of spermatogenesis which becomes lower. Thus, the decrease in testes volume indicates a histological structure disorder. Similar results showed that the decrease in gonad volume was correlated with the abnormalities of the gonad function.[11]

Decreasing diameter of seminiferous tubule is followed by hyalinization of the basal membrane. Progressive hyalinization could be an inhibitor between peritubular capillaries and seminiferous tubule epithelium. This condition would accelerate atrophy of germinal cells.[12] Hyalinization was unclear, however, that is common as this was due to hormonal mechanism disorder at the hypothalamus-hypophysis-testes axis through two ways i.e. hypogonadotrophic hypogonadism and hypergonadotrophic hypogonadism.[12] Hypogonadotrophic hypogonadism affects the hypothalamus and hypophysis, while hypergonadotrophic hypogonadism was an expression of tubular disgenesis. This study shows that damage on histological structure were hyalinization of seminiferous tubule basale membrane and spermatogenesis disorder is demonstrated by low spermatogenesis score due to the effect of a series of combination of TE and DMPA injection through hypothalamus-hypophysis-testes hormonal axis. In relation to hormone levels in the serum, the level of free testosterone and estradiol increased during TE and DMPA injection. This could force the feedback system at hypothalamus and hypophysis, and influence secretion of FSH and LH hormone. Results of this study are also in line with Sutyarso's study on cynomolgus macaques, which stated that injection of combination of TE and DMPA will increase free testosterone hormone in serum. This condition will also result on secretion of LH and FSH by hypophysis.[13] Therefore, the injection of TE and DMPA has a direct effect on LH and FSH levels and an indirect effect on testes function disorder.

The TE influence on reproductive activities in the body will stay for 1.5 months while DMPA, for 3 months.[13] The TE and DMPA injection did not have influence after that period. Therefore after week 30, it is considered as the recovery period. Data show that testes volume, diameter of semiferous tubule, and score of spermatogenesis increase after week 30. This indicates that influence on both spermatogenesis and hormonal reproduction levels is reversible. This happened to both cynomolgus macaques[13] and volunteers (men).[7,8]

CONCLUSIONS

Injection of TE 32 mg and DMPA 40 mg can decrease testes volume and influence testes histological structure, i.e. decreased diameter of seminiferous tubules, basale membrane hyalinization and causes spermatogenesis inhibition. This injection also directly increase the level of total testosterone, free testosterone, and estradiol in serum, and also decrease serum FSH and LH. The influence of injection of TE and DMPA combination on spermatogenesis and hormonal reproduction levels are reversible.

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