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Effect of Supplementation of Bovine Placental Powder on Testicular Tissue and Spermatozoa in Aging Rat Model

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ABSTRACT

Background: Aging in the male reproductive system can cause testicular degeneration. Nowadays, regenerative medicine is a developing domain. This study is conducted to describe the potential effects of dried bovine placenta supplementation on the percentage of abnormal spermatozoa, the density of seminiferous tubules luminal content and Leydig cells counts in D-galactose-induced reproductive aging in male rats.

Methods: A total of 15 healthy adult male Wistar rats were used in this study. Rats were randomly divided into three groups, A: healthy control, B: D-galactose treated, C: D-galactose treated and supplemented with 10% dried bovine placenta. The percentage of abnormal spermatozoa, density of seminiferous tubules luminal content and Leydig cells count were determined. One-Way ANOVA was used for statistical analysis.

Result: The average percent of abnormal spermatozoa did not differ. The seminiferous tubule proportion with full luminal density in Group C (55.2%) was higher than Group B (31.8%, P=0.001) but relatively similar to Group A (62%, P=0.513). The seminiferous tubule proportion with medium luminal density in Group C (23.3%) was lower than Group B (34.2%, P=0.016) but relatively similar to Group A (25.8%, P=1.000). The seminiferous tubule proportion with low luminal density in Group C (21.5%) was lower than Group B (34.0%, P=0.016) but relatively similar to Group A (13.4%, P=0.139). The average number of Leydig cells counted did not differ. In conclusion, supplementation of 10% dried bovine placenta improved the density of seminiferous tubules luminal content in D-galactose-induced reproductive aging in male rats.

Key words: Bovine placenta, Density, D-galactose, Regeneration, Seminiferous tubules.

INTRODUCTION

Degeneration is a condition when cells, tissues or organs have decreased their efficiency, which can be caused by aging and diseases. Aging is a process with gradual biological changes that lead to a decrease in physiological function (Azman and Zakaria, 2019). In male reproductive system, some factors such as aging, exposure to toxic substances, diabetes and oxidative stress which can lead to testicular degeneration (Cele *et al*., 2017). Testicular degeneration is one example that can cause the decreased of spermatozoa quality and even can cause lack of semen reserves in epididymis by reducing the synthesis of androgen/ testosterone (testosterone deficiency) and cause disorders of spermatogenesis and infertility (Wada *et al*., 2016; Aydin *et al*., 2018). The growth of beef cattle population in Indonesia is increasing with a low trend. One major factor for reduced breeding efficiency is the semen used for artificial insemination on beef cows. The demand of frozen semen is increasing, however older elite bulls could have an aging process and cause testicular degeneration. Extending the productive life of elite bulls is one of the efforts that can be made to overcome the incidence of testicular degeneration.

Regenerative medication using placenta-based ingredients is being extensively studied. Chinese traditional medicine has used dried human placenta as one of the materials to treat infertility. Dried placenta consists of many proteins, fibre, lipid, macro and micro minerals and hormones, such as oestrogen, progesterone, testosterone and growth hormone (Tierra and Tierra, 1998; Phuapradit *et al*., 2000). Dried human placenta is a reservoir for some bioactive molecules such as hormones, protein, lipid, nucleic acid, glycosaminoglycan, amino acid, vitamin and mineral that are believed to have bioactivities that inhibit or delay aging, reduce inflammation, sunburn and oxidation and also known to have no toxic effects (Jang *et al*., 2007). Perusal of literature cited no systematic studies on use of dried bovine placenta as regenerative agents on aging. Hence, the present study was undertaken to evaluate the efficacy of dried bovine placenta on D-galactose-induced reproductive aging in male rats.

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D-galactose is used as a substance to mimic-aging in male rats, as per the method that has been adopted by Salman *et al*. (2016). The efficacy of dried bovine placenta as a regenerative agent was tested based on certain parameters such as: abnormal spermatozoa percentage, density of seminiferous tubule luminal content and Leydig cell counts. Thus, the objective of the research is to describe the potential effects of dried bovine placenta on abnormal percentage of spermatozoa, the density of seminiferous tubule luminal content and Leydig cell count in male rat model of reproductive aging induced by D-galactose.

MATERIALS AND METHODS

This study was approved by the Ethical Committee of Faculty of Veterinary Medicine, Universitas Gadjah Mada with the reference number: 0023EC-FKH/Int.2020 and was done from April-October 2020. A total of 15 two month old male Wistar rats were used and kept in the Practical Animal (PA) Laboratory, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta. The rats were adapted for 1 week in the new environment and maintained with a 12-h light-dark cycle in plastic boxes with fine circulation, ambient temperature ranging between 26.3°C and 33.5°C and room humidity between 39% and 86%. The rats were fed with A.D II pellets and fresh water was given adlibitum. The rats were randomly divided into 3 groups: Group A (healthy control), Group B (D-galactose treated) and Group C (D-galactose treated and supplemented with dried bovine placenta). Group A was treated with aqua sterile as placebo while Group B and C were given with D-galactose (3 mg/kg BW) orally for a period of 6 weeks. Later, Groups C were fed with 10% dried bovine placenta mixed with the feed for 30 days. At the end, all rats were euthanized to collect the required samples needed. Freshly expelled bovine placenta (expelled within 6-h of parturition) was collected from dairy farms located in Yogyakarta, Indonesia. The placenta is then cleaned with sterile water and sliced into smaller pieces, then dried in the hot-air oven (50-60°C) for 10-12 h. The dried placenta was grinded to make it as a powder. A total of ten grams of bovine placenta powder was mixed with 90 g of AD.II pellets in 1:9 ratio (10% of bovine placenta powder in AD.II pellets).

The testes along with the epididymis from the experimental rats were removed from the scrotum. About 10 µL semen from cauda epididymis was collected by using a micropipette, then mixed with 990 µL of NaCl solution. One drop of Eosin-Nigrosine and one drop of semen were mixed together. Then, the mixture was smeared on a clean glass slide, the smears were fixed over the flame of Bunsen burner and observed under the light microscope. The abnormal sperm counts were done thrice and the results were averaged and recorded. The density of the luminal contents of seminiferous tubules and Leydig cell count were examined by using microtome sections stained with Haematoxylin and Eosin. The collected tissue samples were fixed using Bouin's solution for 24 hours and then transferred to 70% alcohol solution until further processing. The fixed sample was sectioned crosswise. The pieces of testicular tissue were inserted into a tissue cassette, coded accordingly, processed by paraffin method, cut and transferred on to slides. The slides were deparaffinized using xylene for 5 min and the slides were rehydrated in ethanol with gradual concentrations for 5 min. Harris hematoxylin was used for incubating the slides for 10 min and rinsed in running water for 10 min. After which, the slides were incubated in eosin solution for 20 min. Finally, the slides were dehydrated in ethanol with gradual concentrations for 3 min, then cleared in xylene for 5 min and finally mounted. The slides were examined, using the light microscope with Optilab ® camera. All the data from Haematoxylin-Eosin stained testicular tissue sections were analysed semi-quantitatively for the density of the seminiferous tubule luminal content and Leydig cells were counted and analysed descriptively. The data of percent abnormal spermatozoa was assessed manually using a light microscope. Calculation was done by counting 100 spermatozoa from the slide under 40× magnification. Each sample was evaluated thrice and the results were averaged. The calculation of the density of the seminiferous tubule luminal content was done by counting 100 seminiferous tubules and determining the contents density. The results of the density of the seminiferous tubule luminal content was categorized into three grades, as full, medium and rare. The number of Leydig cells were counted manually using a light microscope. The calculation was done by counting five view fields with 40x magnification. Statistical analysis was done using One-Way ANOVA with the SPSS programme. P values are considered significant at $p<0.05$.

RESULTS AND DISCUSSION

D-galactose a reducing sugar called aldohexose that occured naturally in the body and in foods but could be transformed into aldose and hydroperoxide (H_2O_2) by a catalyst galactose oxidase catalyst, if present in high levels, resulted in release of reactive oxygen species (ROS), which could cause damage to the normal cells. D-galactose induced aging in the reproductive system by over-production of ROS and free radicals and decreased the activity of antioxidant enzymes that are very much similar to the normal aging process (age-related aging) (Ahangapour *et al*., 2014; Zhang *et al.,* 2016; Azman and Zakaria, 2019). The effect of D-galactose in reproductive organs in male laboratory animals was widely described by many researchers. In addition, Datrianto *et al*. (2021) reported that 3 mg/kg of D-galactose fed orally for 6 weeks reduced the average of weight gain in rats. This might be related to ROS that production by the supplementation of D-galactose, due to the activation of NADPH oxidase (Bo-Htay *et al*., 2018). ROS would be formed as the result of activation of glucose mitochondrial oxidative metabolism (Volpe *et al*., 2018). Manna and Jain (2015) reported that ROS could exert some effects on neurons of the hypothalamus that controlled hunger behaviour and satiety.

Spermatozoa abnormalities count

The average percentage of abnormal spermatozoa in Group A, B and C were presented in Table 1. Group A has the highest percentage of abnormal spermatozoa while Group C had the lowest percentage of abnormal spermatozoa. The abnormalities recorded were loose heads. Statistical analysis of percentage of abnormal spermatozoa revealed non-significant difference between the groups (*P*>0.05) (Fig 1). The present observation was contrary with the findings of a previous study (Liao *et al*, 2016) which reported that abnormal sperm morphology was increased in D-galactose-induced mice. Liao *et al.* (2016) used 100-200 mg/kg D-galactose injected intraperitoneally, for 6-8 weeks. The variation in the results of the present study, might be due to variation in the dosage of D-galactose, which was used 3 mg/kg, administered orally for 6 weeks. Further, the differences in percentage of abnormal spermatozoa might be related to the duration of administration of D-galactose, the rate of supplementation and the quality of diet given. Spermatozoal abnormalities caused by D-galactose induction resulted in variation of nine RNA transcripts that were spermatogenesis-related genes, such as Katnb1, Cycl2, Csnka2ip, Zpbp2, Hk1, Pltp, Cabyr, Utp3 and Speer2, which either increased or decreased by at least two times. Some of these genes were essential to maintain the sperm-head morphology and formation or maintain the nuclear integrity. D-galactose induced rats showed lower levels of superoxide dismutase (SOD) and affected the spermatogenic related genes (*e.g.*, Cylc2 and Katnb1) that increased the abnormal morphological sperm and decreased the sperm count (O'Donell *et al*., 2014; Liao *et al*., 2016).

Table 1: Abnormal spermatozoa (%) in Group A, B and C.

Rat sample number	Spermatozoa abnormalities count (%)		
	A	B	C
1	6.0	5.0	5.3
2	2.7	2.3	3.3
3	15.0	7.3	2.3
4	2.7	2.3	-
5	-	6.0	3.0
Average	6.6±5.8[a]	4.6±2.2[a]	3.5±1.3[a]

Different superscript within the row vary significanty (P<0.05).

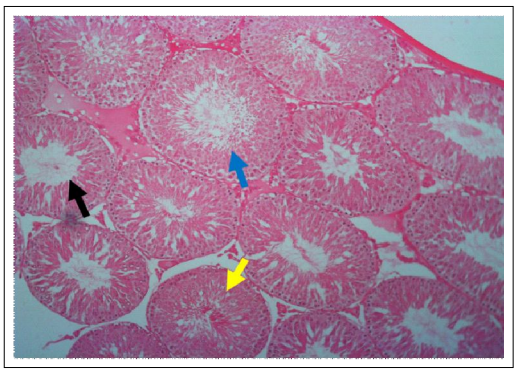

Fig 1: Histology of seminiferous tubules showing full lumen density (yellow arrow), medium lumen density (blue arrow) and rare lumen density (black arrow) (HandE staining).

Seminiferous tubules luminal content density

The average proportion of full, medium and rare density of seminiferous tubule luminal content in Group A, B and C was presented in Table 2. The categories of full, medium and rare seminiferous tubules luminal content density were explained as in the Fig 1. The proportion of seminiferous tubules with full lumen density in Group C (55.2%) was higher compared to Group B (31.8%, P=0.001) but relatively similar compared to Group A (62%, P=0.513). The proportion of seminiferous tubules with medium lumen density in Group C (23.3%) was lower compared to Group B (34.2%, P=0.016) but relatively similar compared to Group A (25.8%, P=1.000). The proportion of seminiferous tubules with rare lumen density in Group C (21.5%) was lower compared to Group B (34.0%, P=0.016) but relatively similar compared to Group A (13.4%, P=0.139). The statistical analysis of the seminiferous tubule content density showed significant difference between the groups (P<0.05). Previous study (Azman and Zakaria, 2019) reported that D-galactose caused damage to the spermatocyte and spermatid in the lumen of seminiferous tubules, decreased number of spermatogenic cells, especially spermatocytes in the lumen of the seminiferous tubules corroborated with the present results. In the present study, the average proportion of full

Table 2: The proportion of seminiferous tubules lumen density categories in Group A, B and C.

Rat sample number	Full (%)			Medium (%)			Rare (%)		
	Group A	Group B	Group C	Group A	Group B	Group C	Group A	Group B	Group C
1	63	40	46	26	32	25	11	28	29
2	53	24	54	34	36	24	13	40	22
3	65	28	64	28	41	18	13	31	18
4	59	38	38	20	32	32	21	30	30
5	70	29	57	21	30	26	9	41	17
Average (mean±SD)	62.0±6.4[a]	31.8±6.9[b]	55.3±7.5[a]	25.8±5.7[cd]	34.2±4.4[c]	23.3±3.6[d]	13.4±4.6[e]	34.0±6.0[f]	21.5±5.4[e]

Different superscript among the means of the same category vary significantly (P<0.05).

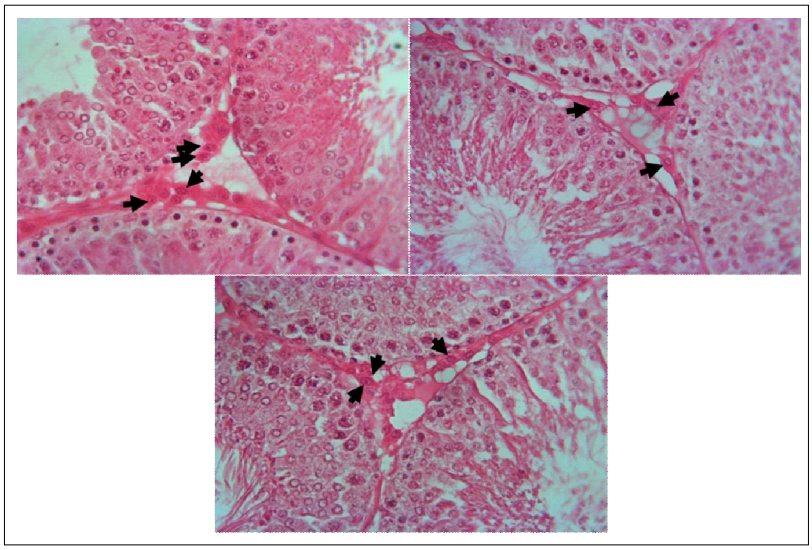

Fig 2: Histology of interstitial tissue showing the Leydig cells (black arrow) (HandE staining).

Table 3: Leydig cells count in testicular tissue among Group A, B and C.

Rat sample number	Group A	Group B	Group C
1	38	35	45
2	55	34	52
3	61	41	52
4	48	28	34
5	45	44	36
Average (mean ± SD)	49.4±9.0[a]	36.4±6.3[a]	46.3±7.6[a]

Different superscript within the row vary significantly (P<0.05).

lumen of seminiferous tubule content density of Group A and C were significantly higher than Group B. The average proportion of medium density of seminiferous tubule lumen content of Group B was significantly higher than Group A and C. The average proportion of rare seminiferous tubules lumen content density of Group B was higher than Group A and C (Fig 2). Prihatno *et al*. (2021) also stated that the administration of D-galactose could decrease the epididymal sperm count and the supplementation of dried bovine placenta could improve the epididymal sperm count. The testicular lobules consisted of seminiferous tubules that served as the site of spermatogenesis. Jeremy *et al*. (2017) also reported similar results and cited that D-galactose administration could lower daily sperm production and sperm count.

Leydig cell count

The average number of Leydig cells in Group A, B and C were presented in Table 3. Group A had the highest Leydig cell count. Group B had the lowest Leydig cell count. The Leydig cells were presented in Fig 2. Statistical analysis for Leydig cells counts showed no significant difference between the groups. However, the average of Leydig cell count in Group B was lower compared to Group A and C (Fig 2). D-galactose could cause reduction in number of Leydig cells in seminiferous tubules and caused inhibition of steroid biosynthesis by the Leydig cells. Increased levels of ROS caused an imbalance of oxidative stress and antioxidant enzyme activity and might have lead to deficit steroid hormone formation (Ahangapour *et al*., 2014). Tumour necrosis factor (TNF)-alpha, a cytokine present in placenta, might haave maintained the Sertoli cells and germ cells that are important to stimulate and maintain spermatogenesis (Loveland *et al*., 2017). Other cytokines from placenta include, Interleukin (IL) -1, IL-6 and IL-10, could have regulated the Sertoli cells and spermatogenic cell development to maintain an immune tolerance from the immunosuppressive attributes (Hedger and Meinhardt, 2003; Mittal and Roche, 2015).

CONCLUSION

In conclusion, treatment with D-galactose @ 3 mg/kg BW orally for 6 weeks in male Wistar rats could decrease seminiferous tubule lumen content density. Supplementation of 10% bovine placenta powder for 30 days in D-galactose-induced rats could improve the seminiferous tubule contents density.

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Conflict of interest: None.

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