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Blood biochemical parameters levels vary with spermatogenesis in seasonal reproductive model the mink (*Mustela vison*)

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Abstract

Glucose is an essential energy source for germ cells. The fatty acids in the spermatozoa have been suggested to be important for the viability, maturity, and function of spermatozoa. The amount of cholesterol in the seminiferous tubules is also known to be inversely correlated with spermatogenic activity. However, these substances are little or not produced in the testis and must be imported from the bloodstream. In this study, we analyzed changes in selected blood biochemical parameters to determine whether there is a link between them and the spermatogenic activity variation in normal mink (*Mustela vison*) and in mink with spontaneous autoimmune orchitis (AIO) in which spermatogenesis is absent. Our results showed that glucose levels were significantly ($p < 0.005$) lower during the breeding season compared to out of the breeding season in normal mink. In mink with AIO, glucose level was 10 times higher ($p < 0.001$) compared to normal mink in March. TC and TG showed no significant changes. HDL-C levels were lower and LDL-C levels higher during the breeding season compared to out of it in normal mink. HDL-C and LDL-C showed no significant changes in AIO compared to normal mink. The results showed here suggest that the blood levels of certain biochemical parameters are influenced by spermatogenesis in mink.

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Introduction

Biochemical parameters such as lipids and carbohydrates play an important role in reproduction and fertility. Cholesterol is crucial for the development of germ cells, the fertility of spermatozoa as well as for the testosterone production. Previous studies have reported that cholesterol required for spermatogenesis is imported from blood lipoproteins (Akpovi *et al.*, 2006; Fofana *et al.*, 2000). Glucose, another essential biochemical parameter involved in spermatogenesis, is used by Sertoli cell (Robinson and Fritz, 1981) and germ cells (Nakamura *et al.*, 1984). In recent years, there has been growing interest in the use of the mink, an animal model which exhibits seasonal reproductive activity characterized by changes in spermatogenic activity during the annual seasonal reproductive cycle, in studying infertility. The literature provides much information on biochemical blood parameters in mink (Harrington *et al.*, 2012; Rui *et al.*, 2012). Certain biochemical parameter (Glucose, HDL-C, LDL-C) levels were reported higher in blood during the reproductive season as compared to the non-breeding season, while other decrease (triglyceride, total cholesterol) (Lasota *et al.*, 2014). It was shown that several factors affect these parameters, including age and sex of the animal, gestational status, stress, season of the year, etc (Hunter, 1996). However, it is not known whether spermatogenesis influences circulating levels of biochemical parameters in mink. Although cholesterol and glucose required for spermatogenesis are imported from bloodstream, it is not known if blood levels of these parameters vary with the spermatogenic activity.

The reproductive cycle in the adult mink is divided into the 12 months of the year and includes a period of spermatogenic activity which maximum is in February and March, and a period of inactivity characterized by testicular regression from April to July (Pelletier, 2011). Moreover, spontaneous autoimmune orchitis (AIO) reported in mink (Tung *et al.*, 1981 and 1984) is characterized by the absence of spermatogenesis and infertility. These characteristics made the mink suitable for the analysis of changes in

blood biochemical parameters during spermatogenesis in normal mink and in mink with AIO. We hypothesize that blood lipid parameters (total cholesterol, triglycerides, HDL cholesterol and LDL cholesterol) and glucose levels vary during spermatogenesis in normal mink. Therefore, this study was conducted to investigate possible link between spermatogenesis activity and biochemical parameter levels in blood. knowing that liver contributes significantly to circulating levels of lipid and glucose control (Ishida, 2005), we also chose Aspartate amino transferase (AST) and alanine amino transferase (ALT) as markers to investigate liver damage in normal and in mink with AIO.

Material and methods

Animals

Male mink (*Mustela vison*) purchased from Visonnière (St. Damase, Qc, Canada) were individually caged with food, water, and natural lighting. A total of 70 mink were used: 60 normal adults (5 adult for each month) and 10 adults with AIO (5 adults for February, and March). Anesthesia was carried out by intraperitoneal injection of 0.9 ml/kg body wt of phenobarbital sodium (Somnotol; MCI Pharmaceutical, Mississauga, ON, Canada) and 0.15 ml/kg of a solution of 0.3 g/ml chloral hydrate and blood was obtained by cardiac puncture (Pelletier *et al.*, 2009). The protocol was approved by the University of Montreal Animal Care Committee.

Normal fertile male. Blood samples were collected from 2- to 3-year-old fertile adults the last week of every month of the annual reproductive cycle. The dynamic of the germ cell population during development and the annual reproductive cycle are depicted in Fig. 1A. The germ cells were identified by the method of Pelletier (Pelletier, 1986).

Infertile mink with AIO. Black and sapphire (genetically related to black) mink were used. Males that did not sire a litter following their first mating season in March due to primary infertility were excluded from the study (Tung *et al.*, 1981). Only mink of 2–3 yr that mated and sired five or more

litters in the previous year but were sterile during the current year and diagnosed with secondary infertility due to spontaneous AIO were employed.

Clinical criteria of fertility. Criteria for fertility tests were described and serum samples from the same mink used by Pelletier *et al.* (2009) were choosing for this study. Briefly, the ejaculated semen recovered from vaginal lavage was evaluated under the light microscope and the morphology, motility and the number of spermatozoa were assessed for each male mink in March. In addition, anti-sperm antibody levels in serum were measured (Pelletier *et al.*, 2009). Only mink with low sperm counts or immobile spermatozoa, high antibody levels, and histopathology of the testis with leukocyte infiltration and destruction of the seminiferous epithelium at autopsy were diagnosed with secondary infertility of immunological etiology.

Serum samples collection

Serum samples were obtained from anesthetized animals by cardiac puncture. The blood was collected directly into dry tubes without anticoagulant. Blood collections took place between 8.00 am and 10.00 am, before feeding. The blood was centrifuged within 30 min to 60 min after the start of the samplings, and the serum was aliquoted into 1.5 ml Eppendorf tubes and stored frozen at -80°C until time of analysis.

Measurement of biochemical parameters

Total cholesterol (TC) (kit from Biolabo, France), high density lipoprotein cholesterol (HDL-C) (kit from Biolabo, France) and triglycerides (TG) (kit from ELITech Group, France) were assayed by enzymatic methods. Low density lipoprotein cholesterol (LDL-C) was determined using the Friedewald formula. For free cholesterol (FC) and esterified cholesterol (EC) determination, TC and unesterified cholesterol were measured in serum with an enzymatic kit (Wako Chemical USA, Inc., Richmond, VA) as previously described (Akpovi *et al.*, 2006). EC levels were obtained by subtraction of FC from TC. EC and FC contents are expressed in mg cholesterol/dL serum. AST and ALT levels were determined using an

automated blood analyser Hitachi 705 (Hitachi, Japan), using DiaSys (Diagnostic Systems GmbH, Germany) reagents.

Statistical analyses

Data were analysed by SigmaPlot statistical analysis software 2010 (Systat Software, Inc. San Jose, CA, USA). Means and standard errors of the mean (SEM) of blood parameters were calculated. Student's t-test ($\alpha=0.05$) was used to ascertain any difference between the group characteristics.

Results

Normal mink

In adult mink, the active spermatogenic phase occurs in January-March and breeding takes place in the second and third weeks of March. This is followed by an "inactive" spermatogenic phase characterized by a reduction of the mitotic and meiotic activities and by disappearance of spermatozoa and later by a period when spermatogonial stem cells actively divide (A_{od}) in August (AUG), the month used as the hallmark of the onset of the seasonal active spermatogenic phase (Pelletier, 1986; Pelletier *et al.*, 2009).

Blood glucose

Blood glucose was at its maximum level from August to January, out of the breeding season (Fig. 1B). It decreased in February ($p<0.005$) and in March ($p<0.005$) where the minimum level was reached. However, this was followed by a significant ($p<0.002$) increase from April to July (Fig. 1B).

Aspartate amino transferase (AST), and alanine amino transferase (ALT)

AST enzyme activity level was elevated in August to October but decreased significantly ($p<0.005$) in November and December (Fig. 1C). This decrease was followed by a significant ($p<0.002$) increase in January where the maximum level of AST activity level was reached. In February, the level of AST activity decreased again ($p<0.005$) to the level of December followed by an increase in March ($p<0.05$) to the level of the beginning of the reproductive cycle in August. ALT activity level was low from August to

December but increased significantly ($p < 0.05$) in January (Fig. 1C). In February, ALT activity level decreased ($p < 0.0001$) to the level of August-December and stayed unchanged until the end of the reproductive cycle in July (Fig. 1C).

Lipids parameters

Free cholesterol (FC) and esterified cholesterol (EC) did not change significantly throughout the annual reproductive cycle (Fig. 2A). Total cholesterol, measured by automated blood analyser, also showed no variation during the annual reproductive cycle in normal mink (Fig. 2B). Triglycerides level did not vary significantly from August to June but increased

significantly ($p < 0.05$) at the end of the reproductive cycle in July (Fig. 2C). The level of LDL-C was higher than the level of HDL-C throughout the annual cycle. Both LDL-C and HDL-C levels showed no changes from August to January. In February, the level of LDL-C increased significantly ($p < 0.05$). In March however, the level of HDL-C decreased significantly ($p < 0.001$) while that of LDL-C stayed higher. HDL-C level increased in April ($p < 0.05$) and showed no changes from May to July. In opposite to HDL-C, the level of LDL-C decreased in April ($p < 0.005$) and in May ($p < 0.01$) followed by an increase in June ($p < 0.05$) and a decrease in July ($p < 0.005$) (Fig. 2C).

Table 1. Biochemical parameters in normal mink and in mink with AIO serum.

Measured Parameters	February		March	
	Normal	AIO	Normal	AIO
Glucose	0.41 ± 0.12	$0.82 \pm 0.71^+$	0.20 ± 0.08	$1.87 \pm 0.34^{++}$
TC	260.14 ± 38.89	297.89 ± 79.55	234.00 ± 29.58	211.33 ± 70.32
TG	54.00 ± 9.12	61.33 ± 10.01	58.75 ± 9.53	46.33 ± 8.96
HDL-C	76.00 ± 9.71	71.51 ± 23.07	46.75 ± 8.38	57.67 ± 18.02
LDL-C	183.48 ± 24.43	212.93 ± 62.01	190.75 ± 24.36	145.60 ± 58.88
ALT	40.33 ± 5.92	$85.00 \pm 23.64^{**}$	38.50 ± 8.58	$98.33 \pm 29.95^*$
AST	43.67 ± 6.81	$153.67 \pm 51.73^{\#}$	59.00 ± 8.19	$160.00 \pm 7.18^{**}$

Glucose (g/L), total cholesterol (TC) (mg/dL), triglycerides (TG) (mg/dL), high density lipoprotein cholesterol (HDL-C) (mg/dL) and low density lipoprotein cholesterol (LDL-C) (mg/dL) as well as the enzymatic activity of alanine aminotransferase (ALT) (U/L) and asparagine aminotransferase (AST) (U/L) were determined in normal and AIO mink serum. Five independent experiments were carried out per group of animal. The values are expressed as means \pm SEM (* $p < 0.05$; ** $p < 0.005$; # $p < 0.02$; + $p < 0.01$; ++ $p < 0.001$: AIO vs Normal of the same month).

Mink with AIO

In AIO, blood glucose level was significantly higher ($p < 0.01$) in February and in March ($p < 0.001$) compared to normal mink (Table 1). The enzyme activity of both ALT and AST was significantly higher in February ($p < 0.005$ and $p < 0.02$ respectively) and in March ($p < 0.05$ and $p < 0.005$ respectively) in AIO compared to normal mink. No significant difference was found between AIO and normal mink for total cholesterol, triglycerides, TC, HDL-C and LDL-C levels in February and in March (Table 1).

Discussion

Normal fertile male

The aim of this study was to analyse the relative

variations of selected biochemical parameters during the annual and seasonal reproductive cycle in adult male mink, not the absolute values of these parameters. We showed that blood glucose level was higher from April to January, outside the breeding season and lower during the breeding season from February to March. It is reported that the spermatogonia utilise glucose as the major energy substrate (Nakamura *et al.*, 1984), but spermatocytes and spermatids suffer a rapid decline in their ATP content in glucose supplemented media and require lactate/pyruvate for the maintenance of their ATP concentrations (Jutte *et al.*, 1981; Mita and Hall, 1982). In contrast, spermatozoa use glucose/fructose as the major source of energy (Nakamura *et al.*,

1984). During spermatogenesis, the dependence of germ cells on lactate/pyruvate and glucose for energy metabolism keeps changing (Bajpai *et al.*, 1998) and our results suggest that this changing is reflected in blood. ALT activity values were within the range reported by Hunter (1996), from 14.7 to 128.5 U/L. However, our results differed from those reported by Rouvinen-Watt *et al.* (2010) (135 U/L) and by Rui *et al.* (237.71 U/L) (2012). The results of AST activity showed in this article were most similar to the reference values for male Brown mink (53.3 to 60.7 U/L) given by Hunter (1996). Rui *et al.* (2012) observed in Black male mink the level of 229.01 U/L which is more than three times higher compared to our results.

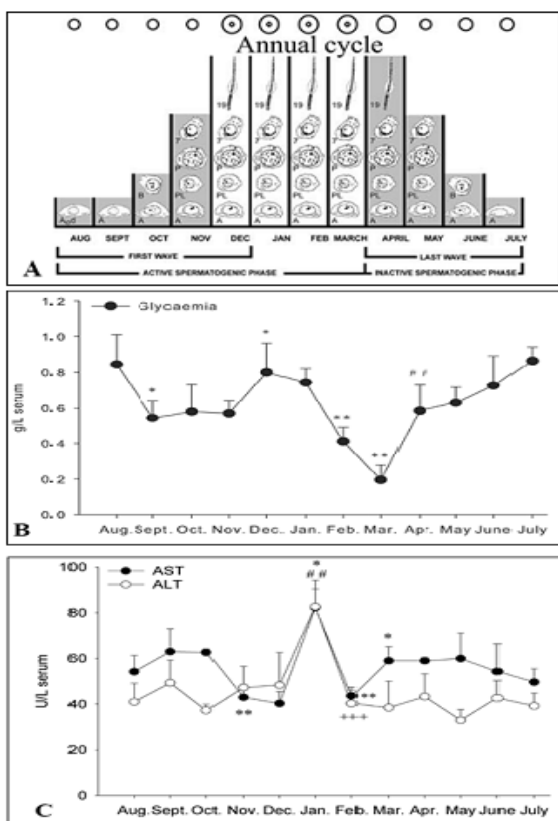


Fig. 1. Diagram showing the oldest generation of germ cells in seminiferous tubule cross sections during each month represented by vertical columns during the annual seasonal reproductive cycle in the adult (A) (Pelletier *et al.*, 2011). The first active spermatogenic phase occurs in January-March. Mink breed during the 2nd and 3rd weeks of March. During the so-called inactive phase that follows, mitotic and meiotic activities are reduced and spermatids disappear. The permeability status of the blood-testis

barrier relates to the presence of a tubular lumen (represented by an inner circle atop each diagram) not to the appearance of any particular generation of germ cells in the epithelium (G: Gonocytes; Pre-A: pre-type A spermatogonia; Aod: Ao spermatogonia dividing; A: type A spermatogonia; PL: pre-leptotene spermatocyte; P: pachytene spermatocyte; 7: step 7 spermatid; 19: step 19 spermatid (Pelletier, 1986; Pelletier *et al.*, 2009; Pelletier *et al.*, 2011). B: Blood glucose levels variation during the annual reproductive cycle in adult mink. The measures were done in serum obtained by centrifugation one hour after sampling. The values shown are means \pm SEM of five independent experiments. C: ALT (open circles) and AST (closed circles) levels were measured in serum during each month of the annual reproductive cycle in five different animals and the results are presented as means \pm SEM. To determine whether changes were significant, the results of one month were compared with results of the previous one by Student's *t*-test (* $p < 0.05$; ** $p < 0.005$; ## $p < 0.002$; + $p < 0.01$; +++ $p < 0.0001$).

To our knowledge, this is the first study reporting on free and esterified cholesterol values in mink serum. We showed that free cholesterol and esterified cholesterol levels, measured by enzymatic method (Wako) did not vary significantly throughout the annual reproductive cycle. More importantly, our results indicate that free cholesterol (73.17 to 106.70 mg/dL serum) and esterified cholesterol (88.41 to 109.76 mg/dL serum) were within the same range of values. Total cholesterol, measured by autoanalyzer, ranged from 178.08 to 251.35 mg/dL serum (4.63-6.53 mmol/l) and showed no significant variation throughout the annual reproductive cycle although testosterone level was reported high in February followed by a sharp drop in March in normal mink (Kabbaj *et al.*, 2003). This indicates that there is no direct correlation between serum testosterone and cholesterol levels. In testis however, infertility in male is associated with increased cholesterol level (Akpovi *et al.*, 2006; Akpovi *et al.*, 2014). Total cholesterol results we showed here are consistent with reference values reported by Lasota *et al.* (2014), 4.61-5.75

mmol/l and by Harrington *et al.* (2012), 3.2-6.8 mmol/l. Triglycerides values showed no significant variation until the end of the reproductive cycle where we found significant increase from June to July. Our result differed from those reported by Lasota *et al.* (2014) who showed that triglycerides level were lower in January-March compared to September-November. HDL-C level showed no significant variation from April to February, decreased significantly in March and increased in April. LDL-C level variation was similar to that of HDL-C profile except that LDL-C was higher in March and June when HDL-C was lower. The inverse variation of both parameters in March is interesting since the breeding takes place in the second and third weeks of March (Pelletier, 1986).

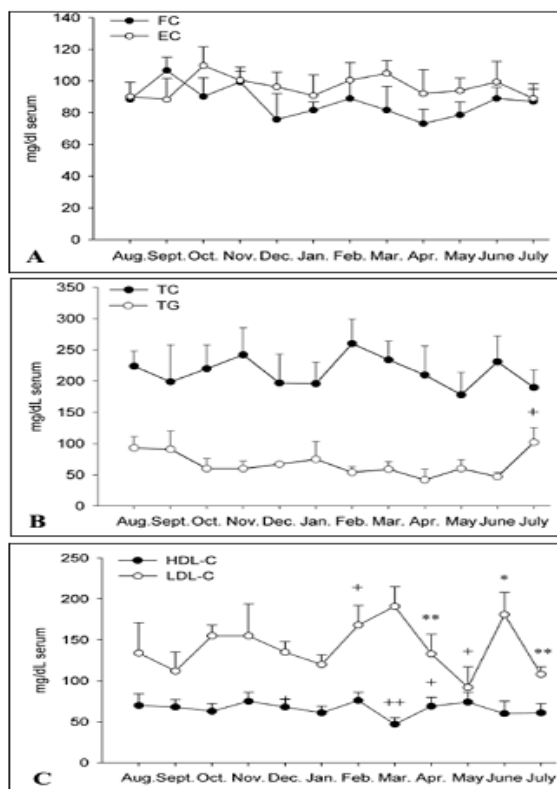


Fig. 2. Free cholesterol (FC) and esterified cholesterol (EC) levels in normal serum throughout the annual reproductive cycle (A). FC (closed circles) and EC (open circles) were determined by enzymatic kit (Wako Chemical USA, Inc., Richmond, VA) and results are expressed in mg cholesterol/dL serum. **B:** Total cholesterol (TC) (closed circles) and triglycerides (TG) (open circles) levels were measured in serum by usual method in clinical laboratory. **C:** High density lipoprotein cholesterol (HDL-C) (closed

circles) and low density lipoprotein cholesterol (LDL-C) (open circles) were analyzed throughout the annual reproductive cycle in clinical laboratory. All values shown are the means \pm SEM of five independent experiments. To determine whether changes were significant, the results of one month were compared with results of the previous one by Student's *t*-test (* $p < 0.05$; ** $p < 0.005$; + $p < 0.01$; ++ $p < 0.001$).

Infertile mink with AIO

Blood glucose level was significantly increased in AIO mink of February and March compared to normal mink. This result highlights our finding in normal mink that glucose level increased out of the breeding season when spermatogenesis is uncompleted. Because AIO mink is characterized by the absence of spermatogenesis (Tung *et al.*, 1984), our results suggest that completion of spermatogenesis in mink is associated with low glucose level in blood. Total cholesterol, TG, HDL-C and LDL-C levels showed no significant changes in AIO compared to normal mink suggesting that circulating cholesterol and TG levels are not fundamentally affected by AIO in mink. In testes however, cholesterol level increased in AIO mink (Akpovi *et al.*, 2006). ALT and AST levels were higher in AIO mink compared to normal mink, however within the normal ranges (Hunter, 1996).

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