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Article

Effect of *Nigella sativa* Seed Supplementation on Hematology, Acid-base Parameters, and Serum Biochemical Parameters in Nubian Goat Fed an Aflatoxin Contaminated Diet

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Abstract: This study aimed to investigate the effect of *Nigella sativa* (*NS*) seed supplementation on hematology, acid-base parameters, and serum biochemical parameters in Nubian goats fed an Aflatoxin-contaminated diet. In a completely randomized design, 20 growing male goats (aged 8-9 months; 11±0.5 kg) were allocated to five treatments (4 goats/treatment). The control group (G1) received a basal diet. The treatment groups received the same diet contaminated with 150 ppb Aflatoxin (G2), and other treatments received an Aflatoxin-contaminated diet supplemented with different levels of crushed *NS* seeds 2% (G3), 4% (G4), and 6% (G5). Blood samples were collected after 40 day feeding period to determine blood pH, glucose, hematological and biochemical parameters. Statistical analysis was performed to assess the significant differences among the treatments. Hemoglobin concentration (Hb), total erythrocytes count (TEC), mean corpuscular hemoglobin (MCH), serum total protein (TP), and globulins (GB) were significantly (P≤0.05) decreased by Aflatoxin-contaminated diet, whereas total leukocytes count (TLC) increased (P≤0.05). Supplementing *NS* seeds to an Aflatoxin-contaminated diet significantly (P≤0.05) increased Hb, TEC, TP, and GB. Lipid profile and serum liver enzymes were significantly (P≤0.05) increased by an Aflatoxin-contaminated diet. Supplementing *NS* seeds to an Aflatoxin-contaminated diet caused a decrease (P≤0.05) in lipid profile and serum liver enzymes. Supplementing *NS* seeds to an Aflatoxin-contaminated diet resulted in a good performance and improved physiological status, the superior effect to an Aflatoxin-contaminated diet supplemented with 6% *NS* seeds. The study recommended supplementing 6% *NS* seeds to goat diets to reduce suspected Aflatoxin contamination. Further investigations are needed to assess the protective effect of *NS* seeds in other animal species fed on Aflatoxin-contaminated diets.

Keywords: Aspergillus, Black seeds, Goat, Hemoglobin, Liver enzymes, Triglycerides.

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1. Introduction

Aflatoxin contamination is a global concern, affecting crops, livestock, and human health on a wide scale, especially in developing countries where inadequate storage and processing methods exacerbate the challenges of ensuring food safety and economic progress. Aflatoxin refers to a group of toxic and carcinogenic secondary metabolites produced by certain strains of *Aspergillus spp*. fungi that can grow on feeds and food products. Aflatoxins are common during the pre and post-harvest stages of feeds, causing adverse effects in different animals and negative economic impacts worldwide, especially in regions with hot and humid climates [1,2]. Many types of Aflatoxins commonly occur in animal feeds and have been considered powerful natural carcinogenic agents in mammals. The maximum limit of Aflatoxin in food and feeds for the consumption of humans and animals is set to 20 ppb by the U.S. Food and Drug Administration [3], and 15 ppb by the European Union [4]. Accurate values of the Aflatoxin concentration that causes Aflatoxicosis have not been confirmed; however, with the help of a few studies, it is estimated that generally, 50–300 ppb of Aflatoxin concentration in feeds can cause Aflatoxin toxicity in animals. Ruminants are more resistant to Aflatoxin than non-ruminant animals because the rumen microbiota can degrade or deactivate toxins [5,6].

Exposure of livestock to feed containing Aflatoxins leads to a broad spectrum of detrimental health impacts, causing notable changes in biochemical, hematological, and performance parameters. Changes in hematological and biochemical parameters occurred

before clinical symptoms developed in chronic and subclinical Aflatoxicosis [7,8]. Significant changes in hematological and biochemical parameters have been observed in Aflatoxicosis cases, which can assist in the diagnosis of toxication [9,10].

Some strategies have been developed to detoxify Aflatoxin contaminated in animal feeds. Physical, chemical, and biological methods have been applied to the removal and biosynthesis of Aflatoxins or as an inhibitory growth factor of Aflatoxigenic molds. However, few of these strategies have practical applications. Therefore, several herbals were tested to reduce the production of Aflatoxin and the growth of molds; one of these herbal plants is *Nigella sativa* [11,12].

Nigella sativa (*NS*), also known as black cumin or black seeds, belongs to the Ranunculaceae herbaceous family. It is primarily found in Mediterranean countries. The seeds of *NS* have been historically utilized for both culinary and medicinal purposes. *Nigella sativa* contains beneficial plant secondary metabolites in the principal component, thymoquinone, which shows antioxidant activities, including other valuable attributes. Dietary supplementation of *NS* may have favorable effects on nutrient intake, nutrient digestibility, growth and milk performance, and reproductive performances, along with improving immunity status and gut health of small ruminants. In addition, crude oil extracts of *NS* showed anticancer, antioxidant, antimicrobial, antipyretic, analgesic, and anti-inflammatory properties [13,14]. It has been investigated that *NS* supplementation inhibited fungus *Aspergillus* growth and thus reduced Aflatoxins synthesis. However, few studies are available on the protective effect of *NS* seeds against Aflatoxin and its effects on animals' performance and physiological status. Dietary supplementation with *NS* increased goats' hemoglobin concentration and total erythrocyte count [15]. Supplementation of *NS* increased serum total protein, albumin, and globulin while decreasing triglycerides and cholesterol in lambs [16]. Furthermore, the inclusion of *NS* in rabbit diets resulted in an increase in plasma total protein, albumin, and globulin [17]. *Nigella sativa* alleviates Aflatoxin-induced liver damage by its anti-inflammatory, antioxidant, and antiapoptotic effects. Therefore, *NS* can be used as a feed additive to alleviate any potential Aflatoxin toxicity from contaminated diets [18].

Nigella sativa supplemented diet may have a protective property against the development of aflatoxicosis in farm animals. Therefore, this study aimed to determine the effect of supplementing *NS* seeds to a diet contaminated with Aflatoxin on hematology, acid-base parameters, and serum biochemical parameters of growing Nubian goats.

2. Materials and Methods

2.1. Ethical approval: The Ethical Committee of the Faculty of Animal Production, University of Khartoum, Sudan, approved the animal experiment in this study (ethical number: 1/2017/3).

2.2. Experimental animals and management: Twenty healthy growing Nubian goats, aged between 8-9 months and with an average body weight of 11±0.5 kg, were distributed randomly to five treatments (four goat/treatment). The goats were purchased from a local livestock market and allowed one week of the feed adaptation period. The growing goats received prophylactic treatment, including 20mg/kg of Oxytetracycline administered intramuscularly for five days, 20mg/kg of ivermectin administered subcutaneously to address ectoparasites, and 10mg/kg of Albendazole administered orally to treat endoparasites. The growing goats were housed individually in well-ventilated open-sided experimental pens (1.5 m × 1.5 m × 2 m). The pens were treated with cypermethrin spray as an anti-parasitic agent and disinfected with 40% formalin. Manual feeding and drinking equipment were used.

2.3. Experimental diets: Groundnut cake infected with Aflatoxin was combined in different ratios with other feed items to create treatment diets with around 150 ppb of Aflatoxins. Aflatoxin concentration was determined using high-performance liquid chromatography (HPLC) according to the method described by Sobolev [19]. *Nigella sativa* seeds were ground into medium size particles. The chemical composition of *NS* seeds used was dry matter (DM) 96.3%, ether extract (EE) 30.7%, crude protein (CP) 28.2%, Ash 4.7%, crude fiber (CF) 20.6%, nitrogen free extract (NFE) 12.1% and metabolizable energy (ME) 15.6 MJ/kg. Five isocaloric and isonitrogenous diets were formulated according to the standard nutrient requirements of goats published by the National Research Council, NRC [20]. The ingredients and the chemical composition of the experimental diets are shown in Table 1. The feed ingredients were manually mixed until final homogeneity was achieved in the mash mixture to formulate the experimental diets: **G1**: Control diet (a basal diet without Aflatoxin and *NS* seeds), **G2**: diet contaminated with 150 ppb

Aflatoxin, **G3**: diet contaminated with 150 ppb Aflatoxin + 2% *NS* seeds, **G4**: diet contaminated with 150 ppb Aflatoxin + 4% *NS* seeds, **G5**: diet contaminated with 150 ppb Aflatoxin + 6% *NS* seeds.

The chemical composition of experimental diets was determined on a dry matter basis. The experimental diets were analyzed for dry matter (DM), ether extract (EE), crude protein (CP), Ash, and crude fiber (CF) by the procedure described by the "Association of Official Analytical Chemists" [21]. Nitrogen Free Extract (NFE) was calculated as NFE% = {DM – (EE% + CP% + CF% + Ash %)}. Metabolizable energy (ME) was calculated according to MAFF [22]: ME for Ruminants (MJ/Kg) = 0.12 CP + 0.31 EE + 0.05 CF + 0.14 NFE. The growing goats were fed experimental diets for 40 days and allowed unlimited water access.

Table 1. Ingredients and chemical composition of the experimental diets

Ingredients %	**Experimental diets groups**				
	G1	**G2**	**G3**	**G4**	**G5**
Sorghum grain	43	43	42	43	42
Groundnut cake	10	10	10	9	8
Groundnut hull	25	25	27	27	27
Wheat bran	20	20	17	15	15
Limestone	1	1	1	1	1
NaCl (salt)	1	1	1	1	1
Nigella sativa	0	0	2	4	6
Aflatoxin (ppb)	0	150	150	150	150
Chemical composition, %DM					
Dry matter	94.17	94.16	94.31	94.8	94.8
Ether Extract	2.17	2.16	2.41	2.9	2.6
Crude protein	16.6	16.3	16.5	16.6	16.7
Crude fibre	9.6	9.3	9.7	9.4	9.8
Ash	12.6	12.2	12.3	12.7	12.6
Nitrogen free extract	53.2	54.2	53.4	53.2	53.1
ME (MJ/Kg)	10.5	10.1	10.7	10.8	10.7

G1: control diet. G2: diet with 150 ppb Aflatoxin. G3: diet with 150 ppb Aflatoxin + 2% *NS* seeds. G4: diet with 150 ppb Aflatoxin + 4% *NS* seeds. G5: diet with 150 ppb Aflatoxin + 6% *NS* seeds. ppb: part per billion. DM: dry matter. ME: Metabolizable energy.

2.4. Collection of blood samples: Blood samples were collected at the end of the feeding experimental period on day 40 at 9:00 am before feeding. The hair in a specific neck area was closely trimmed, and the region was disinfected with a 70% ethanol solution before performing a jugular vein puncture. Five milliliters of blood were drawn using disposable plastic syringes. Following the withdrawal, 1 milliliter of the blood sample was transferred into a capped test tube containing ethylene-diamine-tetra-acetate (EDTA) as an anticoagulant for hematological analysis. The remaining blood samples were left at room temperature for 1-2 hours and then subjected to centrifugation (Gallenkamp junior, Germany) at 3000 revolutions per minute (r.p.m.) for 15 minutes to separate the serum. The samples were pipetted into clean vials and promptly frozen at -20°C for subsequent serum analysis.

2.5. Hematological parameters: The hematological parameters (total erythrocyte count - TEC, total leukocyte count - TLC, mean corpuscular volume - MCV, mean corpuscular hemoglobin - MCH and mean corpuscular hemoglobin concentration - MCHC) were determined according to the methods described by Schalm's Veterinary Hematology [23]. Hemoglobin concentration was determined by the cyano-methemoglobin method using Drabkin's solution, as described by Van Kampen and Zijlstra [24]. Hematocrit (Hct) levels were determined using plain capillary tubes (Umedic, Germany), according to Weiss and Wardrop [25].

2.6. Blood and serum parameters: Blood glucose level was determined by the enzymatic method using a kit (Biosystem, Spain), according to Trinder [26]. Blood pH was promptly measured using a pH meter (HANNA instruments, Portugal) immediately after collection. Serum sodium [Na^+] and serum potassium [K^+] levels were determined using a flame photometer technique (PFP7 Jenway, E.U). Serum chloride [Cl^-] concentration was determined using a commercial kit (Spinreact, Spain). Serum strong ion difference [SID_3] was calculated using the equation described by Constable et al. [27]: Serum [SID_3] (mmol/l) = ([Na^+] + [K^+] - [Cl^-]).

Serum total protein was determined using a Biuret method according to Ohnishi and Barr [28] using kits (Spain react, Spain). Serum albumin was determined using the bromocresol green (BCG) method as described by Doumas et al. [29] using a commercial kit (Spain react, Spain). Serum globulins concentration was calculated by subtracting the serum albumin concentration from the serum total protein concentration. Serum urea concentration was determined by the colorimetric method [30]. Serum creatinine concentration was determined by a colorimetric method described by Henry [31]. Serum total lipids were measured using the method described by Frings and Dunn [32]. Serum triglycerides concentration was determined by the enzymatic method using a kit (Biosystem, Spain), according to Fossati and Prencipe [33]. Serum cholesterol concentration was determined using an enzymatic method with the assistance of a commercial kit (Biosystem, Spain), according to Svensson et al. [34]. The concentration of serum low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were determined by a precipitating reagent using the method described by Friedman and Young [35] and Tietz et al. [36].

Serum Aspartate transaminase (AST) and Alanine aminotransferase (ALT) activity were measured using the UV enzymatic method with the aid of a commercial kit (Liner chemical, Spain) according to the method described by Reitman and Frankel [37]. Alkaline phosphatase (ALP) activity was determined spectrophotometrically according to the method described by Moss et al. [38]. Serum Gamma-glutamyltransferase (GGT) activity was determined spectrophotometrically according to the method described by Szasz [39].

2.7. Statistical analysis: Statistical analysis was performed using SPSS computer program (version 20). Analysis of variance (ANOVA) was used to assess the difference between the treatments. The significance of differences was examined by Duncan's multiple-range tests [40]. The mean difference was considered significant at $P \leq 0.05$.

3. Results

3.1. Hematological parameters: Hematological parameters of growing Nubian goats fed an Aflatoxin-contaminated diet supplemented with *NS* seeds are presented in Table 2. Hemoglobin concentration (Hb) displayed a significant variation among the experimental groups. The group fed an Aflatoxin-contaminated diet (G2) exhibited the lowest Hb value, which was significantly lower ($P \leq 0.05$) compared to the other groups. Supplemented %6 *NS* seeds to an Aflatoxin-contaminated diet (G5) caused a significant increase in Hb ($P \leq 0.05$) compared to the other *NS* supplemented groups.

Total erythrocyte count was significantly ($P \leq 0.05$) decreased in the Aflatoxin-contaminated diet group (G2). Supplementing different levels of *NS* seeds to an Aflatoxin-contaminated diet showed a significant increase in TEC; the higher value was observed in the group fed an Aflatoxin-contaminated diet supplemented with 2% *NS* seeds (G3). Aflatoxin-contaminated diet increased the total leukocyte count. Significantly ($P \leq 0.05$), the highest value of TLC was observed in the group fed an Aflatoxin-contaminated diet (G2), followed by the group fed an Aflatoxin-contaminated diet supplemented with 2% *NS* seeds (G3). On the other hand, the control group and the groups supplemented with 4% (G4) and 6% (G5) *NS* seeds exhibited a significant ($P \leq 0.05$) decrease in TLC.

There were no significant ($P > 0.05$) differences among the groups in Hct, MCV, and MCHC. The group fed an Aflatoxin-contaminated diet (G2) had a significantly ($P \leq 0.05$) lower mean MCH value than the other groups. Additionally, when *NS* seeds were supplemented at a level of 6% (G5), there was a significant increase ($P \leq 0.05$) in MCH compared to the other groups with different *NS* supplementation levels.

Table 2. Hematological parameters of growing Nubian goats (n=20) fed an Aflatoxin-contaminated diet supplemented with *Nigella sativa* seeds.

Parameters	Experimental groups					SEM	SL
	G1	G2	G3	G4	G5		
Hb (g/dl)	10.0 ^{ab}	8.4 ^{b}	9.7 ^{ab}	9.7 ^{ab}	11.3 ^{a}	0.32	*
TEC ($\times 10^6$ /µl)	10.5 ^{ab}	8.7 ^{b}	12.0 ^{a}	10.0 ^{ab}	10.0 ^{ab}	0.40	*
TLC ($\times 10^3$ /µl)	9.3 ^{c}	17.0 ^{a}	13.5 ^{b}	9.7 ^{c}	9.7 ^{c}	0.73	*
Hct (L/L)	0.23	0.26	0.27	0.29	0.29	0.01	N.S
MCV (fl)	22.5	31.1	23.2	29.1	29.3	1.31	N.S
MCH (pg)	7.9 ^{b}	6.7 ^{c}	8.2 ^{b}	7.9 ^{b}	11.2 ^{a}	0.48	*
MCHC (g/dl)	35.6	37.7	35.2	33.1	39.3	1.25	N.S

G1: control group. G2: group fed a diet with 150 ppb Aflatoxin. G3: group fed a diet with 150 ppb Aflatoxin + 2% *NS* seeds. G4: group fed a diet with 150 ppb Aflatoxin + 4% *NS* seeds. G5: group fed a diet with 150 ppb Aflatoxin + 6% *NS* seeds. Hb: Hemoglobin. TEC: Total erythrocytes count. TLC: Total leukocyte count. Hct: Blood hematocrit. MCV: Mean Corpuscular Volume. MCH: Mean Corpuscular Hemoglobin. MCHC: Mean Corpuscular Hemoglobin Concentration. SEM: Standard error of the means. SL: Significance Level. N.S: Non-significant. *: P≤0.05. abc: Means with different superscripts in the same row were significantly different.

3.2. Blood glucose, serum protein parameters, urea, and creatinine: Blood glucose, serum protein parameters, urea, and creatinine of growing Nubian goats fed an Aflatoxin-contaminated diet supplemented with *NS* seeds presented in Table 3. Blood glucose levels showed non-significant (P>0.05) differences among the experimental groups. There were significant (P≤0.05) variations among the experimental groups on serum total protein. Significantly (P≤0.05) lower mean value of TP was observed in the group fed an Aflatoxin-contaminated diet (G2) compared to other groups. Supplementation of *NS* seeds caused a significant increase (P≤0.05) in serum TP levels. Notably, the group fed an Aflatoxin-contaminated diet supplemented with 6% *NS* seeds (G5) exhibited a significantly higher (P≤0.05) value of TP compared to the other *NS* supplemented groups. No significant (P>0.05) differences were recorded among the groups on serum albumin. However, a significantly (P≤0.05) lower value of serum globulins was observed in the group fed an Aflatoxin-contaminated diet (G2) compared to the other groups. Conversely, the group fed an Aflatoxin-contaminated diet supplemented with 6% *NS* seeds (G5) displayed a significantly higher (P≤0.05) value of serum globulins compared to the other *NS* supplemented groups. The results obtained in the present study indicate no significant (P>0.05) differences among the groups on kidney function, as presented in creatinine and urea nitrogen concentrations, which were not affected by the supplementation of *NS* seeds to an Aflatoxin-contaminated diet. However, a slight increase in urea level was observed in *NS* supplemented groups.

Table 3. Blood glucose, serum protein parameters, urea, and creatinine of growing Nubian goats (n=20) fed an Aflatoxin-contaminated diet supplemented with *Nigella sativa* seeds.

Parameters	Experimental groups					SEM	SL
	G1	G2	G3	G4	G5		
Glucose (mg/dl)	42.5	42.2	42.5	42.7	43.2	0.74	N.S
Total protein (g/l)	45.0 ^{c}	36.0 ^{d}	45.0 ^{c}	67.0 ^{b}	80.0 ^{a}	0.38	*
Albumin (g/l)	25.0	22.0	21.0	24.0	26.0	0.10	N.S
Globulins (g/l)	20.0 ^{c}	13.0 ^{d}	24.0 ^{c}	43.0 ^{b}	54.0 ^{a}	0.37	*
Urea (mg/dl)	55.0	55.2	58.5	61.0	66.2	2.2	N.S
Creatinine (mg/dl)	0.85	0.97	0.82	0.85	0.87	0.42	N.S

G1: control group. G2: group fed a diet with 150 ppb Aflatoxin. G3: group fed a diet with 150 ppb Aflatoxin + 2% *NS* seeds. G4: group fed a diet with 150 ppb Aflatoxin + 4% *NS* seeds. G5: group fed a diet with 150 ppb Aflatoxin + 6% *NS* seeds. SEM: Standard error of the means. SL: Significance Level. N.S: Non-significant. *: P≤0.05. abc: Means with different superscripts in the same row were significantly different.

3.3. Blood pH, serum electrolytes, and strong ion difference: Blood pH, serum electrolytes, and strong ion difference of growing Nubian goats fed an Aflatoxin-contaminated diet supplemented with *NS* seeds are presented in Table 4. The results indicate that all groups showed no significant differences (P>0.05) in terms of blood pH, serum sodium [Na^+], serum potassium [K^+], serum chloride [Cl^-], and serum strong ion difference [SID_3].

Table 4. Blood pH, serum electrolytes, and strong ion difference (SID_3) of growing Nubian goats (n=20) fed an Aflatoxin-contaminated diet supplemented with *Nigella sativa* seeds (mmol/l).

Parameters	Experimental groups					SEM	SL
	G1	G2	G3	G4	G5		
Blood pH	7.35	7.35	7.30	7.35	7.30	0.01	N.S
Serum [Na^+]	141.0	142.2	142.7	142.5	141.0	0.45	N.S
Serum [K^+]	5.07	4.82	4.40	4.55	4.72	0.08	N.S
Serum [Cl^-]	103.7	107.7	108.5	108.5	102.7	1.11	N.S
Serum [SID_3]	42.3	39.3	38.6	38.5	42.9	1.17	N.S

G1: control group. G2: group fed a diet with 150 ppb Aflatoxin. G3: group fed a diet with 150 ppb Aflatoxin + 2% *NS* seeds. G4: group fed a diet with 150 ppb Aflatoxin + 4% *NS* seeds. G5: group fed a diet with 150 ppb Aflatoxin + 6% *NS* seeds. SEM: Standard error of the means. SL: Significance Level. N.S: Non-significant. abc: Means with different superscripts in the same row were significantly different.

3.4. Lipid profile: Significant (P≤0.05) differences were observed among the groups in serum lipid profile parameters, as presented in Table 5. A higher (P≤0.05) total lipids concentration was observed in the group fed an Aflatoxin-contaminated diet (G2) compared to the other groups. However, supplemented *NS* seeds to an Aflatoxin-contaminated diet led to a significant (P≤0.05) decrease in serum total lipids. Specifically, supplementing 2% *NS* seeds to the Aflatoxin-contaminated diet (G3) resulted in a significantly (P≤0.05) lower value of total lipids compared to the other groups with different supplementation levels.

Table 5. Serum lipid profile of growing Nubian goats (n=20) fed an Aflatoxin-contaminated diet supplemented with *Nigella sativa* seeds.

Parameters	Experimental groups					SEM	SL
	G1	G2	G3	G4	G5		
Total lipids (g/dl)	17.3 ^{ab}	21.3 ^{a}	9.2 ^{b}	15.2 ^{ab}	15.1 ^{ab}	1.34	*
Triglycerides (mg/dl)	14.0 ^{ab}	19.2 ^{a}	6.7 ^{cd}	12.0 ^{bc}	5.7 ^{d}	1.36	*
Cholesterol (mg/dl)	38.5 ^{b}	49.2 ^{a}	34.7 ^{c}	36.5 ^{c}	29.0 ^{d}	2.70	*
HDL (mg/dl)	26.5 ^{b}	30.7 ^{a}	22.5 ^{c}	25.0 ^{b}	19.5 ^{a}	1.90	*
LDL (mg/dl)	6.0 ^{b}	12.4 ^{a}	6.2 ^{b}	5.5 ^{b}	3.5 ^{c}	0.39	*

G1: control group. G2: group fed a diet with 150 ppb Aflatoxin. G3: group fed a diet with 150 ppb Aflatoxin + 2% *NS* seeds. G4: group fed a diet with 150 ppb Aflatoxin + 4% *NS* seeds. G5: group fed a diet with 150 ppb Aflatoxin + 6% *NS* seeds. HDL: High-density lipoprotein. LDL: Low-density lipoprotein. SEM: Standard error of the means. SL: Significance Level. *: P≤0.05. abc: Means with different superscripts in the same row were significantly different.

Serum triglyceride level was significantly (P≤0.05) highest in the group fed an Aflatoxin-contaminated diet (G2) compared to the other groups. Notably, supplementing *NS* seeds to an Aflatoxin-contaminated diet at a level of 6% (G5) resulted in a significantly (P≤0.05) lowest concentration of serum triglycerides. The experimental group fed an Aflatoxin-contaminated diet (G2) exhibited a significantly (P≤0.05) highest mean value of total cholesterol. Supplementing *NS* seeds to an Aflatoxin-contaminated diet led to a significant (P≤0.05) decrease in serum total cholesterol. Specifically, supplementation of *NS* seeds at a level of 6% (G5) resulted in a significantly (P≤0.05) lower value of serum cholesterol compared to the

other groups. The highest value (P≤0.05) of high-density lipoprotein (HDL) was observed in the group fed an Aflatoxin-contaminated diet (G2). However, supplementing *NS* seeds to an Aflatoxin-contaminated diet caused a decrease (P≤0.05) in HDL, with the lowest mean value observed in the group fed an Aflatoxin-contaminated diet supplemented with 6% *NS* seeds (G5). A significantly higher (P≤0.05) mean value of LDL was observed in the group fed an Aflatoxin-contaminated diet (G2) compared to the other groups. In contrast, the *NS* supplemented groups and the control group showed a significant (P≤0.05) decrease in serum LDL, with the lowest (P≤0.05) value observed in the group fed an Aflatoxin-contaminated diet supplemented with 6% *NS* seeds (G5).

3.5. Serum liver enzymes: Significant (P≤0.05) differences were observed among the groups in all serum liver enzyme values, as shown in Table 6. The results indicate that the AST, ALT, ALP, and GGT values significantly (P≤0.05) decreased by supplementing *NS* seeds to an Aflatoxin-contaminated diet. The experimental group fed an Aflatoxin-contaminated diet (G2), and the group fed an Aflatoxin-contaminated diet supplemented with 2% *NS* seeds (G3) showed significant (P≤0.05) highest values of AST, ALT, and ALP. However, the group fed an Aflatoxin-contaminated diet supplemented with 6% *NS* seeds (G5) had significantly lower (P≤0.05) values compared to the other groups. The Aflatoxin-contaminated diet (G2) significantly (P≤0.05) increased the level of GGT. The addition of 6% *NS* seeds to an Aflatoxin-contaminated diet resulted in a significant (P≤0.05) decrease in GGT levels compared to the other groups.

Table 6. Serum liver enzymes of growing Nubian goats (n=20) fed an Aflatoxin-contaminated diet supplemented with *Nigella sativa* seeds (μ/l).

Parameters	Experimental groups					SEM	SL
	G1	G2	G3	G4	G5		
AST	167.0 ^{b}	178.5 ^{a}	177.5 ^{a}	161.0 ^{b}	147.0 ^{c}	8.04	*
ALT	37.2 ^{b}	68.7 ^{a}	68.2 ^{a}	35.7 ^{b}	29.2 ^{c}	4.90	*
ALP	15.5 ^{b}	21.7 ^{a}	19.7 ^{a}	16.7 ^{b}	13.1 ^{c}	1.06	*
GGT	16.2 ^{c}	23.0 ^{a}	19.0 ^{b}	14.0 ^{c}	9.7 ^{d}	1.42	*

G1: control group. G2: group fed a diet with 150 ppb Aflatoxin. G3: group fed a diet with 150 ppb Aflatoxin + 2% *NS* seeds. G4: group fed a diet with 150 ppb Aflatoxin + 4% *NS* seeds. G5: group fed a diet with 150 ppb Aflatoxin + 6% *NS* seeds. AST: Serum Aspartate Transaminase. ALT: Serum Alanine Aminotransferase. ALP: Serum Alkaline Phosphatase. GGT: Serum Gamma-Glutamyltransferase. SEM: Standard error of the means. SL: Significance Level. *: P≤0.05. abc: Means with different superscripts in the same row were significantly different.

4. Discussion

Aflatoxin contamination is a known risk in feed ingredients such as groundnuts, corn, and cottonseed. The findings of this study provide valuable insights into the impact of Aflatoxin exposure and the potential benefits of *Nigella sativa* seed supplementation on the physiological performance of growing Nubian goats.

The present study findings on hematological parameters, specifically Hb concentration and TEC, agree with previous research conducted by Oguz et al. [41], Abdel-Wahhab et al. [42], and Yousef et al. [43], who reported similar decreases in Hb concentration and TEC as a result of consuming an Aflatoxin-contaminated diet. These findings align with the current study and provide additional support for the adverse effects of Aflatoxin exposure on hematological health. Dietary supplementation with different levels of *NS* seeds significantly increased the Hb concentration and TEC. These findings are consistent with the studies conducted by El-Saadany et al. [15] and Habeeb and El Tarabany [44], which reported similar results of increased Hb and TEC in lactating goats when supplemented with *NS* seeds. The results obtained in the present study for TLC showed a significantly highest value in the group fed an Aflatoxin-contaminated diet (G2), which attributed to the cytotoxic effects of Aflatoxin on a variety of cells, including hematopoietic precursor cells and lymphocytes [45]. The TLC was significantly decreased by supplementing different levels of *NS* seeds to an Aflatoxin-contaminated diet due to the anti-inflammatory properties of *NS* and its active principle, thymoquinone [46].

The results obtained in the present study demonstrate that supplemented *NS* seeds to an Aflatoxin-contaminated diet had no significant effect on blood glucose levels. This result agrees with the findings of Awadallah [47], who reported that the supplementation of *NS* does not significantly impact blood glucose levels in Friesian calves. Furthermore, the results are consistent with the findings of Omer [48], who observed that the supplementation *NS* to the rabbit ration did not affect blood glucose levels.

Serum total proteins and globulins concentrations increased significantly by supplemented *NS* seeds to an Aflatoxin-contaminated diet. The increased concentration of total protein and its fractions can be attributed to the enhanced activity of hepatic functions as a result of *NS* seed supplementation. The significant increase in globulins concentration observed indicates a good immune status of the growing goats. The results obtained from the study indicate that supplementing the diet of the growing goats with *NS* seeds improved immune function and restored physiologically relevant levels of protein function. The results of the present study agreed with Awadalla and Gehad [49], who found that supplementing the rations of growing sheep with 2% *NS* seeds significantly increased total protein and globulin concentrations, while albumin concentration was not significantly affected. Tousson et al. [50] reported increased blood total protein and albumin concentrations in rabbits fed diet containing *NS* seeds. Moreover, El-Saadany et al. [15] reported that supplementation with *NS* improved animal immune function and increased total protein and globulins concentrations. Zeweil et al. [17] found that the addition of *NS* to rabbit diets resulted in increased levels of plasma total protein, albumin, and globulins. Similarly, Habeeb and El Tarabany [44] found that dietary supplementation with *NS* in Zaraibi goats significantly increased serum total protein and globulins. The previous studies agree with the findings in the present study.

Blood pH, serum electrolytes (Na^+, K^+, and Cl^-), and strong ion difference (SID_3) were not affected by an Aflatoxin-contaminated diet or supplementation with different levels of *NS* seeds. These findings disagree with the study conducted by El-Saadany et al. [15], which reported that dietary supplementation with *NS* significantly increased the concentrations of Na+, K+, Ca+, Pi, and Zn in lactating goats. Additionally, the present study's results are inconsistent with the findings of Habeeb and El Tarabany [44], who reported that serum electrolyte concentrations significantly increased with dietary supplementation of *NS* during the hot summer season.

The study's Serum lipid profile results reveal significant differences among the treatment groups. The group fed an Aflatoxin-contaminated diet showed a significant increase in serum cholesterol and LDL levels. However, the supplementation of *NS* seeds resulted in a significant decrease in serum cholesterol and LDL levels. The present study's findings agree with the results reported by Al-Beitawi et al. [51], who observed that feeding with *NS* seeds reduced plasma cholesterol and triglyceride concentrations and increased plasma HDL concentrations. Previous studies showed that *NS* has a promising effect similar to drugs that reduce serum cholesterol and decrease its atherogenic pathological impact [52,53]. The decrease in triglycerides and cholesterol levels observed in this study may be attributed to active ingredients such as thymoquinone and compounds like monounsaturated fatty acids. These components have been shown to reduce cholesterol synthesis by hepatocytes and decrease cholesterol absorption from the small intestine [54]. El-Dakhakhny et al. [55] concluded that reduced serum cholesterol levels may be attributed to enhanced bile production. The decrease in serum cholesterol levels could be due to the high content of unsaturated fatty acids in *NS* seeds, which stimulate cholesterol uptake by the intestine and can be converted to bile acids through oxidation [56].

Liver enzymes profile (AST, ALT, ALP, and GGT) obtained in the present study show significant differences among the groups. Serum liver enzymes increased significantly in the Aflatoxin-contaminated diet group. However, liver enzyme levels were significantly decreased by supplementing *NS* seeds to an Aflatoxin-contaminated diet. This result disagreed with Awadalla and Gehad [49], who found that supplementing growing sheep rations with *NS* did not affect serum liver enzyme activities and indicated that supplementation *NS* had no adverse effects on liver function. The findings of studies conducted by Rastogi et al. [57] and Karakilcik et al. [58] are consistent with the present study, as they also observed an increase in the activity of ALP with Aflatoxin exposure. Similarly, the present study observed a significant increase in the activities of AST and ALT with an Aflatoxin-contaminated diet. These results agreed with Oguz et al. [59] and Madheswaran et al. [60], who reported that an Aflatoxin-contaminated diet significantly increased AST and ALT activities. The observed increases in AST and ALT levels were directly related to the doses of Aflatoxin.

5. Conclusion

The current study indicates that supplemented *NS* seeds to an Aflatoxin-contaminated diet positively affected blood and serum parameters. *Nigella sativa* supplementation improved hematological parameters such as Hb and TEC, suggesting potential benefits for blood health. It also demonstrated immune-enhancing properties, as evidenced by increased serum total proteins and globulins concentrations. Furthermore, *NS* seed supplementation was associated with reduced cholesterol and LDL levels, indicating a potentially beneficial impact on lipid profile. However, no significant effects on kidney function, blood glucose levels, or electrolyte balance were observed with *NS* supplementation. The results suggest that *NS* seeds may hold promise as a dietary supplement to counteract the adverse effects of Aflatoxin-contaminated diets. Further research is needed to explore the underlying mechanisms and confirm these findings in diverse animals. Supplementing *NS* seeds may successfully replace synthetic detoxifying agents, providing an alternative method to reduce suspected Aflatoxin contamination.

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Article

A pilot study about multiple congenital ocular anomalies in trait Comtois horse breed

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Abstract: The Silver gene is at the origin of the special coat color of the Trait Comtois Horse breed, but also predisposes carriers to Multiple Congenital Ocular Anomalies syndrome.

The objective of this pilot study was to evaluate the awareness and knowledge of Comtois owners about this topic through their response to a questionnaire. After completing it, we offered documentation for them to keep and to refer to when they feel the need.

The majority of the owners were aware about the gene, but still many are confused with its issues. Genetic testing is not systematically done when ocular problems are noticed, but willingness to pay more attention to genotypes is present in the common spirit. By developing a proper reproduction management and a proper genotyping of the Comtois horse breed population, conservation and enhancement of the breed will be possible. In this regard, Silver homozygous equines should not be discriminated, as they may possess desirable traits and may be able to perform as any other horse in their domain of activity. They should instead be encouraged to be mated with their bay peers, in order to obtain heterozygous foals. Genetic testing campaigns are increasingly offered during competitions and open to all categories of horses.

This problematic should not divide breeders, but rather motivate them to work altogether to be able to perpetuate the Trait Comtois horse breed, flagship of the Franche-Comté region.

Keywords: Trait Comtois, Silver gene, multiple congenital ocular anomalies

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1. Introduction

Being preyed on animals, visual perception is important for horses to escape danger. More than that, as they are today a very polyvalent breed, they also need this sense to complete different tasks they might be asked in domains such as fieldwork, sports, or hobbies.

Silver dilution is a dominant trait. This means that a horse requires only one parent to carry and pass on the gene. The silver dilution gene will only alter black pigmented horses (E/e or E/E) and alters the coat by diluting areas of black pigment. It has no effect on red pigmented horses (e/e). The effects of the silver dilution gene can vary greatly. When a uniform black horse is diluted by the silver gene, the mane and tail are lightened. The body is also lightened to a chocolate color, which is often dappled as well. A bay horse carrying the silver gene will usually have a lightened mane and tail, as well as lightened lower legs.

Genetic testing can be very important, as the silver gene is not always expressed. Although a red horse will not be diluted by the silver gene, it can be a carrier and pass the gene to its offspring. Because the gene is dominant, only one copy is needed for the horse to develop the silver colorations.

For the Genotype and Phenotype there are multiple possibilities that can result according to the multiple genes involved in color patterns:

- Black with single silver (E/_ a/a Z/N): black silver with iridociliary cysts
- Black with double silver (E/_ a/a Z/Z): black silver with MCOA
- Bay with single silver (E/_ A/_ Z/N): bay silver with iridociliary cysts
- Bay with double silver (E/_ A/_ Z/Z): bay silver with MCOA
- Chestnut with single silver (e/e Z/N): chestnut with iridociliary cysts
- Chestnut with double silver (e/e Z/Z): chestnut with MCOA

Comtois horses are the first draft horse breed in France. Its popularity never stopped to grow: they appeal to the large public through its gentle character, its conformation and its typical coat color. Its characteristic silver mane and tail shade was selected through the years and is linked with a mutant allele from the locus Silver, that is the gene PMEL17, inducing the dilution of the eumelanin pigment ((black and brown) [1]. Multiple congenital ocular anomalies (MCOA) is an inherited syndrome an inherited condition predominantly involving the anterior segment of the eye. Firstly demonstrated in Rocky Mountain Horse related breeds, such as Kentucky Mountain Saddle horses, Mountain Pleasure horses and Morgan horses [2], but also in unrelated breeds such as miniature horses [3], ponies [4] and Icelandic horses [5] it is also responsible for the Multiple Congenital Ocular Anomalies syndrome (MCOA), consisting in lesions localized in the anterior segment of the eye mainly (iridociliary and/or peripheral retinal cysts, cornea globosa, cataract and iris hypoplasia [2, 6-9].

The Comtois horse does not escape this rule and is thus also predisposed to these anomalies. Many foals are born blind, and mature horses are frequently seen with cornea globosa or cataract [10]. Also, in France the subject of ocular anomalies associated with the Comtois breed is evaluated through scientific surveillance and research [11-13].

However, it is possible to reduce the incidence of the disease through genetic testing and good reproduction management programs. In order to tend to the obtaining of an always healthier and sustainable breed, it comes to the responsibility of breeders to make the right choices. In this way, the goal of our study was to elaborate a questionnaire in order to point out the actual knowledge about this topic among Comtois owners.

2. Materials and Methods

2.1. Recruitment, interviewing of horse owners and questionnaire design

This pilot study was conducted through the collection of answers to a Google Form, counting 21 questions. The questionnaire was drawn up and intended for Comtois horse breeders and owners from France. Prior to its publication, a meeting with the President of the National Association of Trait Comtois horse breed was held on the 17th of June 2021 in Besançon, in order to present the project and the goals.

The questionnaire was launched in the first of August 2021 on internet and social media. A QR code leading to our questions when scanning it was also created. The responses collected were anonymous in this situation. On the other hand, breeders and owners were also personally invited to complete this survey which was presented at events such as local, regional and national Comtois breed competitions. The Google Form was closed on the first of March 2022, meaning that the study was conducted over a seven-month period.

Our main goal was to raise the awareness about the Silver gene and its implication in sight problems that might be encountered in Comtois horse. This questionnaire allowed us to realize about the popularity of this subject within the owners population. We also wanted to explain that breeding management is essential in order to avoid or decrease the obtaining of horses with ocular anomalies, since genetic stands for an important part in this field. Finally, we wished to provide an overview and an update about the topic to the members of the Comtois horse association who are implied in the improvement of the breed.

2.2. Questionnaire: documentation

At the end of the questionnaire, we joined two documents that explain the basics of genetics as well as a cross board for the owners to understand better what they can expect from mating when they know the genotype from both partners. In this way, it shows them that avoiding obtaining homozygous foals is possible and not that complex when we know the genotype of both parents.

3. Results and discussion

We obtained 245 answers, a single questionnaire response per stud farm was permitted in this study. *In this first part of the questionnaire*, we wanted to draw the profile of people owning Comtois horses. The five first questions were about general information, such as the department of origin, the time by which they owned Comtois horses and how many of them they have, but also about their enrollment in the breeding program and the domains in which they are using their animals.

- The first question was about the French department of origin of the answering person. We drew the map of France with its different departments and the corresponding number of questionnaire response for each. We also added a scale of blue color shades to be even more readable (Fig.1). What is immediately striking is the number of answers coming from the Franche-Comté region, with a total of 118 answers, that represents 48% of the sampling. The highest rate is located in the department of Doubs with 83 feedbacks, which is not surprising considering that the cradle of the breed is situated in Maîche. The fact that the Comtois horse is the first breed of draft horse in France can be clearly appreciated through the map representation, with answers coming from regions far from its origin. We also received a response from a person living in Switzerland. We chose to include it in our study since this country is neighboring Franche-Comté.

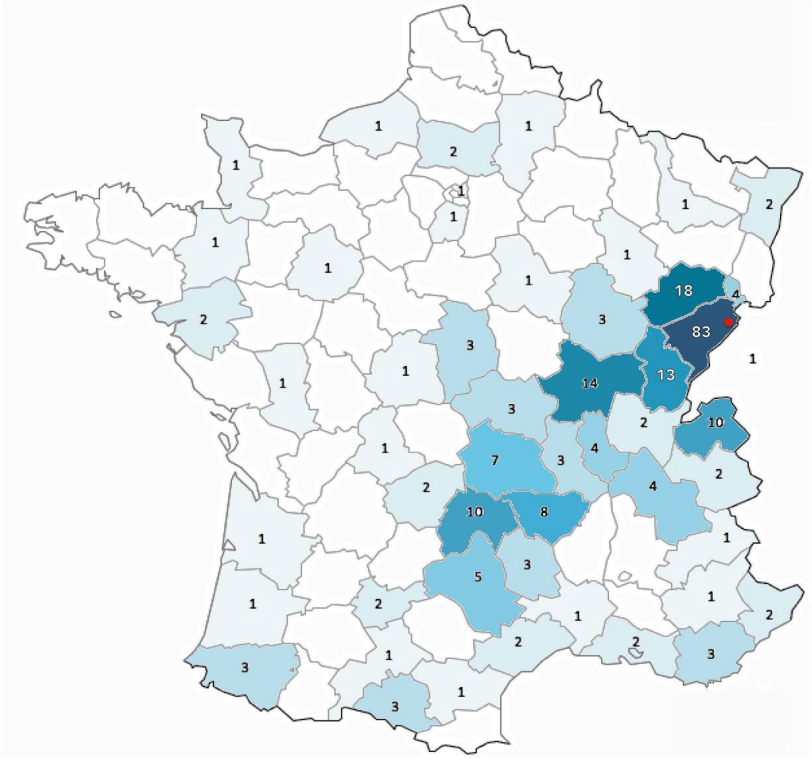

Figure 1. Number of answers to the questionnaire per French department.

- The second question was about how long the person was breeding or owning Comtois horses. All the three time period categories proposed were quite well represented. We can tell that new breeders or owners are the most represented in this study, with a rate of 42.4% of answers. This is an opportunity to cross this statement with their actual knowledge about the Silver gene and its implication in Comtois horse. The consideration of this group is important since it could make it possible to raise awareness of the problem as soon as they enter the world of Comtois horse. This would result in a better education about reproductive management, which will partly lead to the improvement and preservation of the breed. We will also be able to make the comparison with the more experienced persons.

Table 1. Overview of the number of males and females owned in the same stud farm.

Number of females owned	Number of males owned	Number of owners	Corresponding percentage
1 to 5	1 to 5	48	19.6 %
6 to 10	1 to 5	15	6.1 %
11 to 15	1 to 5	11	4.5 %
16 to 20	1 to 5	4	1.6 %
> 20	1 to 5	3	1.2 %
11 to 15	6 to 10	1	0.4 %
16 to 20	6 to 10	1	0.4 %
> 20	6 to 10	1	0.4 %
16 to 20	11 to 15	2	0.8 %
> 20	11 to 15	1	0.4 %
6 to 10	16 to 20	1	0.4 %
> 20	> 20	3	1.2%
		Total=91	Total=37%

- The third question was about the number and sex of the horses owned. According to the results, 54% owned females only, the general tendency was owning between 1 and 5; 9% owned males only, all of them owning between 1 and 5.

37% owned males and females, the general tendency was owning between 1 and 5 horses of each sex (Tab.1).

One person answered by owning no male and no female, this is the reason why the sum of the number of persons owning horses is equal to 244 and not 245. The rest of the data linked with this case were discarded because it was irrelevant for the study. Even if this person has owned Comtois horses in her career, we are rather interested in people owning some in order to have a true overview about what we can observe currently. Thus, we will continue this study by treating the 244 responses left.

The majority is owning female Comtois. This might be due to the possibility of obtaining a foal each year and thus an economic income, but also because of the ease in managing mares rather than stallions. For people owning both sexes, we can think that they answered the questionnaire in the period they were having a male foal under the mother, for example, as we did not ask about the ages of the animals. Indeed, owning intact males together with females require more installations and management, but this is not impossible. Moreover, we did not ask if the males were castrated or not, in this way owning both sexes is easy. Today, genetic testing is compulsorily done on reproductive males only. The fact that females are very well represented is important, as they are giving birth each year for most of them. They are responsible for a half of the genetic heritage of the foal to be born. Thus, they need to be taken as much in consideration as the chosen stallion when it comes to genotyping. These remarks will be discussed in more detail later.

- The fourth question was about the enrollment into the Comtois horse breeding program. The objective of the breeding program is "to allow the Comtois horse breed to improve the quality of all its representatives while promoting the marketing and development of markets and thus to provide farmers with a good level of remuneration for their products". 57.8% are enrolled in the breeding program and 42.2% are not. More than half of the sample is composed of owners breeding and raising Comtois horses to add an economic value to their activities. The rest are often privates with no economic interests.
- The fifth question concerned the domains in which the horses were performing. Ten different proposals were presented, and several boxes could be ticked in this situation. The results are presented in the following histogram (Fig. 3). Most of the horses are used in reproduction (59%), followed by horse and carriage (48.8%), which shows that this breed kept its basic usefulness until today. We can link it to the previous question, in which we showed that 57.8% of breeders were taking part in the breeding program.

These results are consistent. Horse riding takes also a huge part in the activities of the breed (43.4%), showing that it has evolved from being the farmer helper to a companion animal, used for leisure and hobbies. Through this question, we showed that this draft horse breed is very polyvalent: its uses extend from a horse used in agriculture and reproduction, to a horse appreciated by the large public.

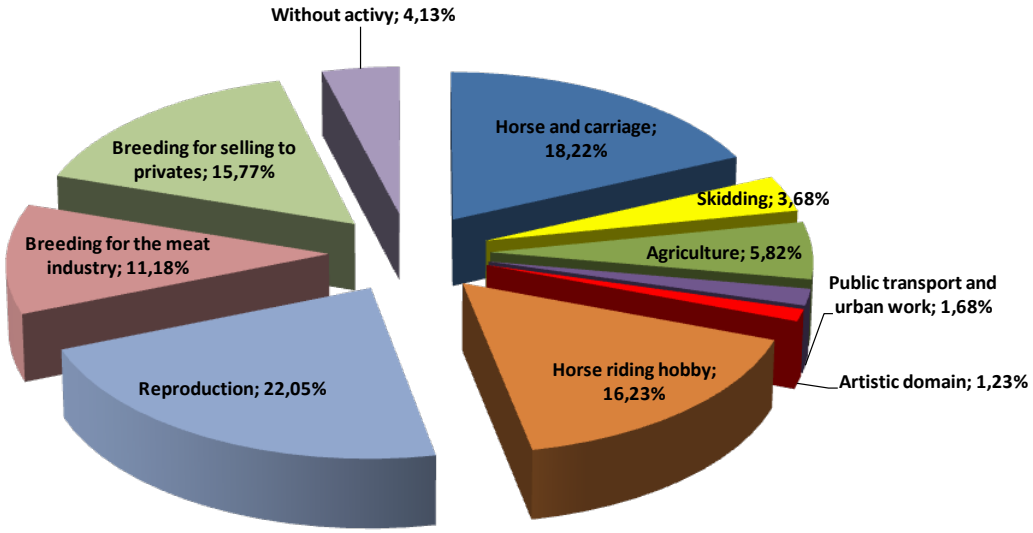

Figure 2. Activity domains of the Comtois horse sample.

The second part of the questionnaire deals with the Silver gene. In this section, we are interested in the knowledge of breeders and owners about this topic.

- The sixth question concerns the knowledge of the existence of the Silver gene. The majority has ever heard about it, which represents a proportion of 73%, which is an encouraging data.

A huge proportion of our sampled owners have heard about the Silver gene. However, when we asked if they were familiar with its implication in the Comtois horse breed, 43.9% were not. In this way, it shows that this subject might be in the mind of many concerned parties but without a real understanding of the issue. From what our discussions with some breeders have shown, this is surely due to the fact that genetics is often perceived as a very specific and complex field, sometimes discouraging them to understand and learn more about this gene.

If we look closely at the portion of new breeders and owners of Comtois horse, meaning less than 5 years of experience, we calculated that 30.2% of them have never heard about this gene and do not know about its implication in the Comtois breed (Tab.2). 44.44% have heard about it and know about its implications, and 25.40% have heard about it but do not really know about its effects. It means that in 5 years of owning this type of horse, at least 69.8% are conscious of the existence of the Silver gene, which is a great progress and shows that prevention and communication about it exists. In contrast, in the category of people owning a Comtois horse for more than 15 years, 25.97% are still not aware about this problematic. We could have expected a smaller percentage in this group because of the experience they are supposed to acquire.

Table 2. Representative table of the experience of owners and their knowledge about the Silver gene.

Owners experience categories and knowledge about Silver gene	Number	Percentage from the considered category	Percentage from the total owners	
Owners with less than 5 years of experience	**63**	**%**	**244**	**%**
Heard about the Silver gene and know about its implications	28	44,44	28	11,48
Heard about the Silver gene but do not know about its implications	16	25,40	16	6,56
Never heard about the Silver gene and do not know about its implications	19	30,16	19	7,79
Owners with 5 to 15 years of experience	**77**	**%**		
Heard about the Silver gene and know about its implications	40	51,95	40	16,39
Heard about the Silver gene but do not know about its implications	17	22,08	17	6,97
Never heard about the Silver gene and do not know about its implications	20	25,97	20	8,20
Owners with more than 15 years of experience	**104**	**%**		
Heard about the Silver gene and know about its implications	67	64,42	67	27,46
Heard about the Silver gene but do not know about its implications	10	9,62	10	4,10
Never heard about the Silver gene and do not know about its implications	27	25,96	27	11,07
Total	**244**			

- After these first two questions of the second part of the questionnaire, we informed our readers that the Silver gene is systematically present in Comtois horses with a clear mane and tail. We added that this gene was responsible for the dilution of the coat color and was predisposing carriers to ocular anomalies. We concluded by specifying that these anomalies were more or less important according to the horse's genotype, meaning homozygous or heterozygous for the Silver gene.
- In the eighth question, we asked them if they were aware that a genetic test existed that allows them to identify if their horse is a carrier. At the same time, the way the test is performed was also exposed. The results were the following, 37.3% did not know about the existence of the genetic test; 33.6% know about the genetic test and that it can be done from blood or horsehair sampling; 29.1% know that a genetic test is possible, but they do not know how it is performed, or more precisely from what sample it is possible to do it. These data demonstrate that 62.7% of people know that genetic testing is available. As for the Silver gene, the parallel should be made directly with genetic testing when raising the awareness of Comtois owners. This way, knowledge about these tests would spread and be easily anchored in the mind of people.

Choosing between hair or blood samples depends on the preference of the person realizing the sampling. When veterinarians are performing it during the national competition on males, blood sampling is the method realized. Also, when harvesting horsehair, caution should be made to get the hair bulb, because it is the only part that contains the genetic information. This last method of sampling is accessible to the large public, as it does not require any particular skill. A form to be completed previously is joined to the hair and send to the laboratory.

The owners were informed them that the average price of a test is around 40€ (at the time the questionnaire was developed). The ANCTC covers these costs for the males when collecting the samples on the national competition of the breed taking place in Maîche in September. For the majority, 68.9%, the answer was positive, for 14.3% it was not, and the 16.8% left were without opinion. The results are promising

because it means that owners would maybe be more ready to perform genetic testing on their horses as they consider it as affordable. Also, group prices are often proposed by laboratories when it comes to many horses to be tested, such as during events. This should further encourage in genotyping.

- In the next question, we asked owners if they had ever used these tests on their animals. We found that 84.8% never did; 7.4% did on some individuals independently of their gender; 4.9% did on stallions only and 2.9% did systematically in mares and stallions. Only 37 persons realized them, meaning 15.2%. We can observe that even if genetic testing is known by 62.7% of people, the proportion of owners that are actually carrying it out is minimal. These results show that more actions should be taken to encourage genetic testing in all animals, independently of their gender. Indeed, there is a ditch between what is known by Comtois owners and their actual actions.

Then, we detailed that in order to be approved to reproduce within the French studbook of the Trait Comtois horse, the stallion candidates must have their genotype determined before the allocation of the first breeding [14]. The results are communicated to the breeder and do not constitute in any case the removal of a stallion from reproduction, whatever its genotype. It comes down to the will and good sense of the breeder to make the proper crosses.

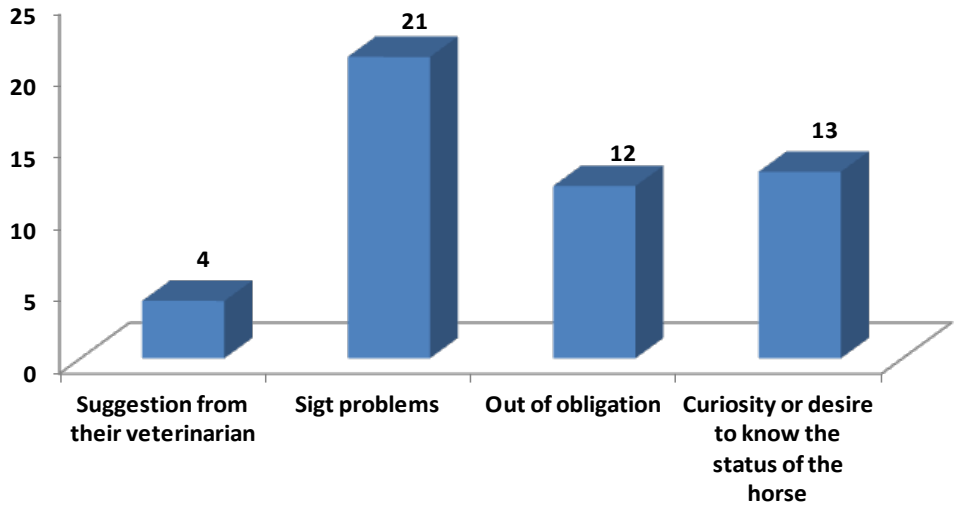

Figure 3. Distribution of owners according to the reasons they wanted to perform genetic testing.

To continue, we wanted to find out for what reasons those 37 persons were realizing genetic testing on their horses. Several boxes could be ticked, and other motivations could be added in this inquiry (Fig.3).

- The last question of this second part was about the importance owners attached to the usefulness of their horse's vision in their domain of activity. We found that 72.1% find it very important; 23% find it important; 1.2% find it of a minor importance; 3.7% are without opinion.

From these last two categories, we checked the domain of activity of the implicated animals in order to understand why these owners do not perceive the sight of their horse as essential in their activities. For the majority, they are used for reproduction only, or they do not have any particular activity. We can think that these persons do not focus on the sight of their horse because it is not primordial in their tasks, as long as they are healthy and can produce a foal each year. However, it is from healthy mares and stallions that they could obtain foals with the best genotype as possible, meaning heterozygous for the flaxen chestnut horses, or non carriers homozygous for the bay ones. For the few other owners remaining, the horses are used for horse and carriage or horse riding. Here, the utility of vision should yet be of first importance.

Sight is considered as an important to very important criterion for the majority of owners. They need to have healthy horses with a sharp sense to better help them in their daily duties or hobbies.

Questionnaire : ocular anomalies

The third part of the questionnaire deals with the ocular anomalies the owners might have encountered in their Comtois horses.

Our fourteenth and fifteenth questions were about the link between the coat color of the horses, namely flaxen chestnut or bay, and the observation of some ocular anomalies they could have such as blindness, big eyes, bad vision from afar, difficulties to locate in dark or heavily lit areas, or others. From our

analysis, we had 11 persons owning exclusively bay horses. From this sample, none declared having noticed ocular problem. We had 110 persons owning exclusively flaxen chestnut horses. From this sample, 45 declared having noticed ocular problems, meaning 40.9%; 120 persons owning flaxen chestnut horses and bay horses, out of this sample 39 never observed ocular problems for both coat colors (32.5%); 54 observed ocular problems in flaxen chestnut only (45%); 2 observed ocular problems in bay only (1.7%); 7 observed ocular problems for both coat colors (5.8%) and the 18 persons left never observed ocular problems or do not know (15%).

Three persons answered by having no bay and no flaxen chestnut horse. We excluded them for the analysis of the data coming out from this question, because it is considered as irrelevant. Indeed, some owners of horses with a particular coat color such as Silver black may have not understood that they should enter the category of flaxen chestnut in this question, as it is the Silver genetic profile we are interested in, generalized as the color of the mane and tail.

Independently of the coat color, 113 persons have observed these kinds of ocular problems in their horses, which represents 46.3%.

As a general fact, bay horses are less represented in the Comtois breed. As they are not carriers of the Silver gene, ocular anomalies reported by owners can not be linked with it. Moreover, they are a minority in this study. On the other hand, the incidence of ocular problems in flaxen chestnut is quite high : 110 persons out of 244 have ever noticed it, which corresponds to 45% of the sample.

We can conclude that most of the owners have ever noticed ocular problems or at least sight deficiency in their horses. This is particularly true for flaxen chestnut Comtois.

In the following question, we asked from these 113 people who noticed ocular problems in their horses if they submitted them to a genetic test. Only 9 of them did, which represents 8% of this sample.

Realizing a genetic test will not help in resolving the vision troubles for the horses that may already have it, but it permits to make a link with their genotype and thus to be able to better apprehend future mating with the aim of decreasing the obtaining of homozygous foals. We will explain it later through the use of a combination square.

We could have added a question concerning the result of the genetic tests for owners who performed it on their horses, but anyway, answers coming from only 8% of the whole sample group would not have been enough representative.

The age at which ocular problems were noticed was asked in the next question. We chose three categories, namely at birth, before two years old or after two years old. We picked the age of two as a reference because it is the time at which males are compulsorily tested during the national competition. Thus, we wanted to know if vision problems were observed by owners before or after this age, trying not to be influenced by the results they could have got from genotyping. It was observed: at birth in 40% of the cases; in horses less than two years old in 31% of the cases; in horses more than two years old in 29% of the cases.

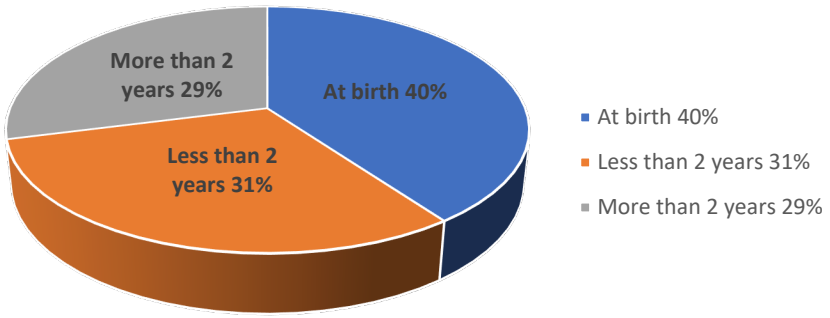

Figure 4. Age at which ocular problems were noticed by Comtois horse owners.

The MCOA syndrome is supposed to be nonprogressive, although it was described earlier that the number of cysts might increase with age but their implication in the impairment of vision is not certified as they are translucent [10].

In most of the cases, sight deficiency or ocular anomalies are noticed early after birth, with sometimes foals reported as being totally blind. The observation of vision problems in horses of more than two years

old might be because the horse was bought at this age or because the horse started to be more handled, enabling the owners to make these observations.

- The last question of this part was about the confirmation of these ocular problems by a veterinarian. For 60% of the owners, it was not confirmed by a veterinarian. From the 40% who had their veterinarian checking the eyes and confirm the ocular deficiency, uveitis was mentioned as a diagnosis from 7% of them. Uveitis represents the inflammation of the uvea, meaning the iris, the ciliary body and the choroid. This disease is not linked with the Silver gene, but can lead to vision loss on a short or a long term if it is chronic. The possible etiologies for uveitis are multiple. We consider it not relevant for our study.

Questionnaire : reproduction

In this last part of the questionnaire, we are approaching the subject of reproduction in Comtois horse, always with the Silver gene consideration.

In the eighteenth question, we asked about the importance breeders and owners were attaching to genotypes in their choices for mating. The vast majority recognize it as being important to very important. From people without opinion, 64% are not using their horse for reproduction. Therefore, this information can in part explain this outcome.

We can criticize these results. Indeed, the majority says that genetics is important in their choice for reproduction, but only a small part of breeders practice genotyping of their Comtois horses, when this is not imposed, as seen in the fifteenth question. One may wonder if there has been confusion with the phenotype of horses used in mating, many choosing stallions according to their personal attractiveness for such or such coat color.

Then, we reminded the fact that bay horses are not carriers of the Silver gene and that consequently, they are not predisposed to the development of ocular anomalies linked to it. This being known, we asked them if they would be ready to choose them more often in their choices for mating.

The survey shows that, 68.4% are ready to do it, 6.6% are not and 25% are without opinion. From our experience and our discussions on the field, because bay Comtois horses are less popular and sometimes less appreciated by some people than flaxen chestnut, it may be a factor explaining the reason why they would not favor them in reproduction.

However, the coat color resulting in the foal from crossing a bay horse to a flaxen chestnut horse will depend on the genotype of the latter. Indeed, for a homozygous, the color obtained will always be flaxen chestnut, as it will inevitably transmit an allele bearing the Silver gene. For a heterozygous, there is a 50% chance to obtain either a bay or a flaxen chestnut foal.

If we analyze the results, from the 11.1% who considered the genotype profiles not that important in their choice for mating, 63% would finally avoid crossing two homozygous horses. It means that this subject may have raised their attention, and they would be ready to consider the best options when it comes to reproduction. In contrast, always considering this pool, 15% would still not avoid crossing homozygous horses and 22% are without opinion.

The overall outcome is satisfying, with almost 70% of people who would pay attention not to mate two homozygous together. We could have added a question for the rest of the owners by asking them why they would not avoid it. We assume that sometimes the phenotype of the horses is more important to the eyes of some persons than the actual genotype of the horses.

For the last question of this study, we were interested in learning how many people were thinking that in addition to knowing the genotype of the stallion, the genotyping of breeding mares would be useful.

Most (77.5%) find it helpful to test mares for the Silver gene as well. This is encouraging, because it shows that breeders and owners are conscious of the problematic and are ready to push further the analyses. In this trend, some local Comtois breed sections are proposing to their members a genetic test on fillies and mares that can be carried out during the cantonal competition. For some, the costs are covered by the section in order to encourage the breeders to participate. This is a good opportunity for them because it would permit a better handling of reproduction by knowing the genotype from both horses and thus make rational choices in order to obtain healthy foals.

4. Conclusions

This section is mandatory but can be added to the manuscript if the discussion is unusually long or complex.

Multiple Congenital Ocular Anomalies syndrome in Comtois horse is caused by a mutation on the Silver locus that codes for the typical diluted coat and hair color of this breed. It predisposes to different ocular anomalies, the severity of which depends on the Silver genotypic profile. The cystic profile corresponds to the development of iridocilliary cysts in heterozygous horses, thought to be less severe than the MCOA profile found in homozygous, in which is added cornea globosa, persistent myosis, iris hypoplasia, cataract, retinal dysplasia and detachment.

Our study goal was to report on the actual knowledge of this problematic in Comtois owners through the use of a questionnaire. We predicted to have some owners with an excellent level of knowledge on this topic, but at the same time we expected that for some of them the understanding of the issues might be difficult, and implementation of the recommendations not always followed.

The existence of the Silver gene is widely known, but the answers were more reserved when we asked if they were familiar with its implication. A bit more than half of the owners know about genetic testing and consider it affordable. However, a minor part actually perform it on their animal and generally out of obligation for stallions. In some cases, ocular problems or desire to know the status of the horse were reasons to realize testing. Since sight was considered as a very important criterion by almost all owners, genotyping could have been expected to be more common. Moreover, about half of them has ever noticed sight deficiencies in Silver bay Comtois. Also, they mentioned that they observed them at birth mainly, and in horses less than two years old. Genetic testing realized on some competition in 2020 and 2021 on young horses showed that half of them are homozygous. This result supports the two previous observations.

Genotype of the horses used in reproduction was considered important by the majority of owners. Yet, this result can be criticized as genetic testing is not enough carried out when it is not compulsory.

After reminding breeders that bay Comtois are not carriers of the Silver gene, two third were ready to use them more often in mating. The same result was obtained when it came to avoid crossing two known homozygous horses together. Finally, more than three quarters of Comtois owners think that it would be useful to genotype mares in addition to stallions.

Sight problems are clearly present in the Trait Comtois breed, and these are noticed by owners. The outcome of this study is encouraging concerning Silver gene management in reproduction. Indeed, in the last part of our questionnaire, after reminding some information, the majority answered with the will to care about the transmission of it.

In the last part presented thereafter, we would like to propose some solutions and some plans to be implemented in the future in order to try to alleviate the occurrence of MCOA syndrome in Trait Comtois horse breed.

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Article

Welfare assessment of the sheltered dogs using behavioral indicators

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Abstract: The aim of this work was to evaluate the welfare of sheltered dogs using behavioral indicators that indicate negative and positive emotional states (provoked and unprovoked behavior). The behavior of the selected animals was evaluated by direct observation of the indicators. The data collected was used for computing a life quality score (LQ). Forty-three behavioral indicators (23 negative indicators and 20 positive indicators) were identified and analyzed in 20 dogs housed for more than two years in the shelter. Six negative indicators (tail chasing, circling, escape attempt, chewing bars, coprophagy and lifting a front leg) were not identified in any of the 20 evaluated dogs. An average LQ score of 0.115 was obtained, with values between -0.35 and 0.4. The results showed that 55% of the assessed dogs had higher LQ scores than the mean value. Canine behavior can be assessed within a reasonable amount of time by recording the presence or absence of certain behavioral indicators. These recordings can then be processed to obtain a quality of life score for each animal.

Keywords: behavioral indicators, dog, life quality score, shelter.

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1. Introduction

Dogs are beloved companion animals throughout the world, but millions of them end up in the care of an animal shelter or rescue organization each year [1]. Divers research have identified that the environment offered by the shelter can have negative effects on the health and well-being of dogs, especially on those who have to live for a long period of time [2 - 6].

In many animal welfare programs, the focus is usually on stress and negative emotional states [3, 7]. However, we must take into account that a complete evaluation must also capture positive emotional states [3, 8]. Several studies have presented certain stressful elements for dogs in shelters: lack of social interaction, little exercise, minimal control over their environment, unpredictable noise levels, and caretaking routines can make living in a shelter stressful to dogs [9, 10, 11]. In a stressful situation, the individual fails to cope and adapt, endangering the well-being of the animal [12]. Welfare assessment in shelter dogs is a very current topic and highly debated both in the mass media and in the veterinary medical world. This is due to the difficulty of defining certain indicators, but also to the complexity of the systems involved and the adaptation skills of individual dogs [13].

Welfare assessment of dogs is based on the use of the ethogram. Most ethograms were created for a specific environment or research area. Thus, based on the ethogram, a series of tools have been validated, which can be generally used in a variety of populations [5, 6, 14, 15, 16, 17, 18, 19].

An ethogram is a list or catalogue of species-specific behaviors. The behaviors are defined and then organized into general categories such as locomotion, resting, playing, change in posture, and stereotypy or repetitive behaviors, of which circling, pacing, whirling, and wall-bouncing are just a few examples. In general, each behavior is recorded along with its frequency and duration in a specific period of time. Once the observation period has ended, the behavior scores are calculated and an assessment is completed [1]. The aim of this work was to evaluate the welfare of sheltered dogs using behavioral indicators that indicate negative and positive emotional states (provoked and unprovoked behavior).

2. Materials and Methods

The study was carried out in a private dog shelter owned by an animal protection association (non-governmental organization) from Cluj county. Animals selected for evaluation had been in the shelter for more than 2 years and at the time of the study had no documented health problems, no ocular discharge, lameness or wounds.

The behavior of the selected animals was evaluated by direct observation based on behavioral indicators indicating negative and positive emotional states (Table 1, Table 2).

The behavioral characteristics were recorded using a binary system 1/0, where 1 has meant the occurrence of a given behavior and 0 has meant its absence, in a given time period, during the assessment that was done [14, 20]. The ethogram used, originally proposed by [14] was adapted to the conditions present in our study. Those characteristics that could not be observed, either due to the design of the shelter or for other objective reasons, were excluded.

The first evaluation method involved videotaping the experiment so that the interaction with the animals could be reviewed later. The purpose of this approach was to increase the precision with which the elements in the ethogram were recorded and to correct any elements possibly omitted by the assessor. This method helped to record the part of unprovoked behavior and the initial interaction between the assessor and the animals. The written evaluation was based on the ethogram. Printed copies were used in the form of a table, which contained the behavioral manifestations to be followed and the names of the dogs to be evaluated. The results were then processed in Microsoft Excel.

The second evaluation was carried out as follows: (1) evaluation of unprovoked behavior and (2) evaluation of provoked behavior.

In the first stage, the evaluator positioned himself 2-2.5 m away from the corner of the box, without trying to interact with the animals in any way. The animals were observed for 3 minutes. Eye contact with the dogs, sudden movements or direct addressing of the animal with commands was avoided during this stage. Unprovoked behavior was recorded by going through the elements of the ethogram and marking with 1 the presence of a certain behavior manifestation and with 0 its absence.

For the evaluation of the provoked behavior the evaluator went to the box and brought the hand close to the fence to allow the dog to know the evaluator. The evaluator then entered the pen and assumed a non-threatening position, crouching next to the fence, allowing the dog to approach. This stage also lasted for 3 minutes. Shy dogs were not called and no attempt was performed to approach them. The results of the ethogram were used to calculate a Life Quality score (LQ score) according to the method proposed by Kiddie and Collins [14, 15].

Table 1. Description and prevalence of negative behavioral indicators

Indicators of negative emotional state, unprovoked behavior		**No. dogs**	**% dogs**
Repeatedly pacing in the pen	Dog repeatedly (>3 times) paces around kennel in a fixed route	9	45
Repeatedly jumping on the kennel wall	Dog repeatedly (>3 times) jumps up kennel wall from one side to another	1	5
Tail chasing	Dog chases its tail repeatedly (>3 times)	0	0
Circling	Dog repeatedly walks around in small circle (>3 times)	0	0
Repeatedly display playing position	Dog repeatedly displays the play bow posture (>3 times)	1	5
Excessive drinking	Dog drinks large volumes of water, in excess of what is normal	0	0
Panting	Dog pants for reasons unrelated to physical exertion or warm ambient temperature (only record if temperature < 25 ^{0}C)	9	45
Apathy	The dog is withdrawn and does not respond to commands	1	5
Escape attempt	Dog attempts to escape kennel in a forceful manner whenever the kennel door is opened	0	0
Hiding	Dog is obscured from view of kennel staff, behind its bed or other kennel furniture for prolonged periods when not asleep (>2mins)	2	10
Chewing bars	Dog repeatedly chews and bites at the bars of the kennel (>20 secs)	0	0
Low posture	Tail is lowered, ears are back and legs are bent	4	20
Coprophagy	Did the dog eat its own or another dog's faeces?	0	0
Lifting a front leg	A forepaw is lifted off the ground and held there	0	0
Standing	Positioned with four feet in contact with ground and legs almost or fully extended	13	65
Sniffing a surface/nose on a surface	The nose is held close to or touching a surface, and/or sniffing the surface	2	10
Whining	High pitched vocalisation	1	5
Aggressiveness toward other dogs	Any lip lifting, growling, snapping, or biting	2	10
Startling	Legs flex briefly, body and head quickly, briefly move back, usually in response to a sudden noise, or dog quickly moves backwards	6	30
Box walking without exploring environment	Travels forward without obviously investigating its environment	2	10
Indicators of negative emotional state, provoked behavior			
Oral behaviors, abnormal movements	Includes tongue out; tip of tongue briefly extended; snout licking; lip licking; swallowing, lip smacking	10	50
Ambivalent posture	A crouched body posture +a position that is higher than the breed-specific position; or a high body posture + by a position of the tail that is below normal	9	45
Aggressiveness	Any lip lifting, growling, snapping, or biting	1	5

Table 2. Description and prevalence of positive behavioral indicators

Indicators of positive emotional state, unprovoked behavior		**No. dogs**	**% dogs**
High level of activity	Increased levels of any locomotion or movement	6	30
Grooming	The dog grooms itself: scratched/washed/stretched	1	5
Alert	Generally inactive but with eyes open, and head and ears moving, can be lying down, sitting or standing	16	80
Scanning the environment	Eyes continuously move to view the environment	19	95
Exploring environment	Walks with nose close to surfaces or sniffing objects	5	25
Adopting playing position	Forequarters are lowered to the ground, with rump raised	1	5
Ears up	Ears held forward	12	60
High body position	Breed specific posture shown by dogs under neutral conditions, but with a higher tail or head elevated and ears forwards, or dog standing extremely erect	10	50
Spending time in the front part of the box	Time spent in the half of the kennel closest to the external wall/door	13	65
Grunting	Isolated intense expiration (breathing out)	3	15
Laying down	Most of body in contact with ground	10	50
Playing with objects	Any vigorous or galloping gaited behaviour directed towards a toy or other object, including chewing, biting, shaking it from side to side, batting it with a paw	0	0
Playing with other dogs	Leaps onto another dog, with body relaxed, stands on hind legs and paws at other dog, places mouth around muzzle, head, neck, or legs of other dog with little pressure, pats another dog with a forepaw, lifting both front paws off the ground rapidly to bounce up and down, done in front of and orientated towards another dog	6	30
Licking other dogs' face	The dog licks the muzzle of its kennelmate	0	0
Tail wagging	Repetitive wagging movements of the tail	12	60
Shaking	Dog shakes its whole body briefly as if drying itself	0	0
Indicators of positive emotional state, provoked behavior			
Tail wagging	Repetitive wagging movements of the tail	13	65
Laying down	Most of body in contact with ground	0	0
Initiating physical contact	Dog starts an interaction with the assessor or kennelmate	13	65
Shaking	Dog shakes its whole body briefly as if drying itself	1	5

3. Results and discussion

Regarding the 43 indicators analysed (23 negative indicators and 20 positive indicators), six negative indicators (tail chasing, circling, escape attempt, chewing bars, coprophagy and lifting a front leg) were not identified in any of the 20 evaluated dogs (Table1, Table 2.).

These indicators were associated in previous studies with high levels of stress or precarious housing conditions [21, 22]. Two of the indicators, namely: tail chasing and circling are considered stereotypic behavioral disorders [23]. Their absence in this study may suggest that the animals are housed in conditions that do not cause the appearance of certain behavioral stereotypes. Also, the lack of these manifestations may suggest that the management practices applied within the shelter have a beneficial effect in preventing negative behaviors that are associated with stress. In our study, the housing conditions were adequate and in conformity with the legal regulations in force. In addition, the boxes were not overcrowded. In some situations, the dogs can be housed in precarious conditions, overcrowded boxes and they can have limited contact with humans [24]. In addition, for some dogs it is possible to be exposed to several traumatic situations, abuse and neglect [25].

Based on the results obtained in the evaluation of the dogs' behavior, a LQ score was calculated for each animal. Thus, an average LQ score of 0.115 was obtained, with values between -0.35 and 0.4. The analysis of the results showed that 55% of the assessed dogs had higher LQ scores than the mean value. Nine animals (D2, D4, D6, D7, D10, D12, D15, D16, D17) had negative scores. The maximum score was recorded for D8 (Figure 1).

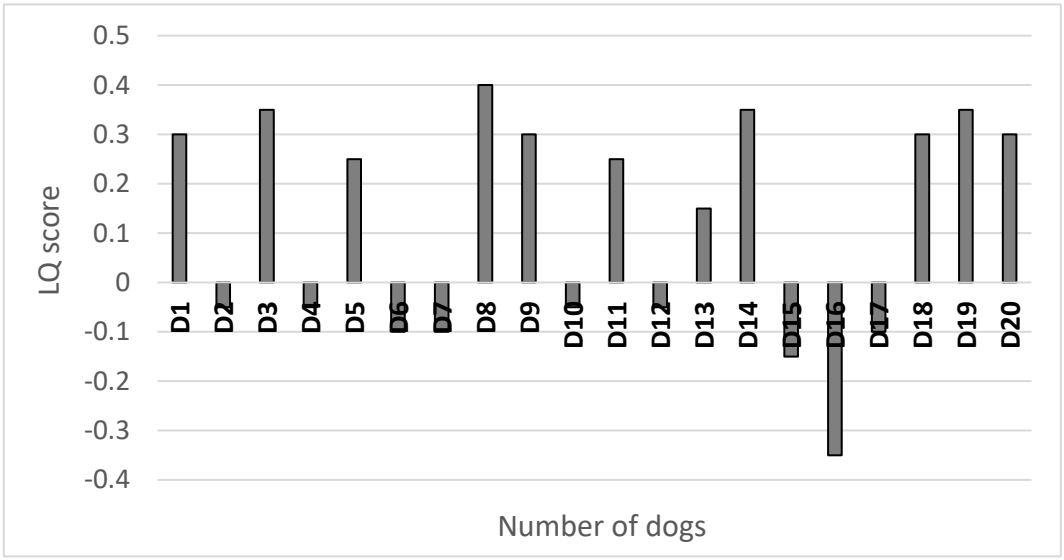

Figure 1. The LQ score obtained by each dog at the evaluation

The maximum percentage of positive indicators (65%) observed was recorded by D14, and the maximum percentage of negative indicators (40%) by dogs D15 and D16. The minimum percentage of positive indicators (10%) was observed in animals D7 and D16, and the minimum percentage of negative indicators (0%) was observed in D3.

The average percentage of positive indicators was 29.25%, more than half of the dogs (55%) exceeding this value. The average percentage of negative indicators was 19.75%, nine of the animals (45%) evaluated registering a lower percentage (Figure 2).

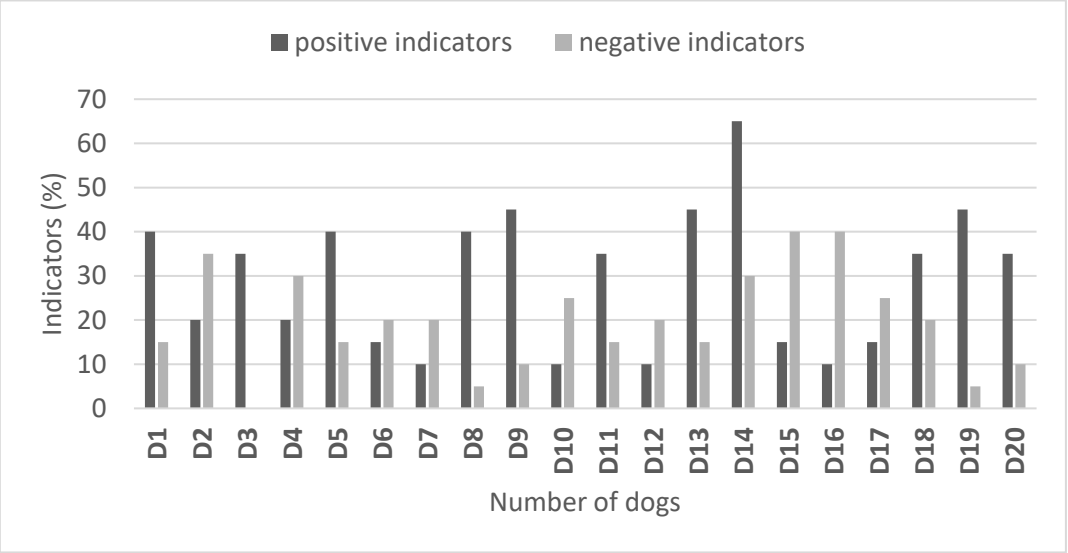

Figure 2. Proportion of positive and negatives indicators per animal

In other studies [14, 15, 20] a percentage of 2% or 30% negative LQ scores were reported compared to 45% obtained in this study.These results suggest a higher number of behavioral disorders in the dogs evaluated in our research. The lower scores obtained in our study could indicate the presence of chronic stress in the dogs or it could be the expression of possible traumas experienced by the animal before entering in the shelter. The dogs in a shelter can be exposed to chronic stress, because several stress factors such as social isolation, changes of the environment, excessive noises, physical restrictions [26, 27].

4.Conclusions

Canine behavior can be assessed within a reasonable time frame by recording the presence or absence of certain behavioral indicators. These recordings can then be processed to obtain a quality of life score for each animal. The quality of life score (QL) is a parameter that can be used to monitor the evolution of the same animal over time, and can also be used to compare the evolution of groups of animals. Additional studies such as the application of socialization programs are needed to gain more knowledge on the behavior of shelter dogs. This aspect is very important especially for animals in shelters that are subjected to a higher level of stress.

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CVJ CLUJ VETERINARY JOURNAL

Review

Decoding the Polyphenol Impact on the Cardiovascular System: A Journey from the French Paradox to Restenosis

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Abstract: Resveratrol and quercetin, two natural polyphenolic compounds, exhibit potential in veterinary and human medicine for cardiovascular benefits, particularly for the prevention and management of restenosis, a complex process involving blood vessel re-narrowing. This review investigates the impact of these compounds on restenosis in animal models, explaining their modes of action, which include antioxidant, anti-inflammatory, and anti-proliferative capabilities. Additional insights are provided by the intriguing "French Paradox," in which the Southern French population's low heart disease incidence is associated with red wine and polyphenol-rich diet consumption. Through animal models, we gain essential knowledge about the therapeutic potential, safety, and dosing of resveratrol and quercetin in both veterinary and human clinical settings. Understanding their precise molecular pathways is essential in enhancing their effectiveness in reducing restenosis. The "French Paradox" draws attention to the potential cardiovascular benefits of polyphenols in restenosis. Novel approaches to minimize restenosis in veterinary and human medicine may result from bridging the gap between animal models and human trials.

Keywords: quercetin; resveratrol, restenosis.

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1. Introduction

Restenosis, the re-narrowing of blood vessels following medical intervention, remains a significant challenge in both veterinary [1] and human medicine [2, 3]. Despite advancements in treatment modalities, the high prevalence of restenosis necessitates further research to develop effective therapeutic strategies. In veterinary medicine, restenosis can occur in various conditions, including coronary artery stenting in companion animals [4, 5] and vascular interventions in porcine models [6, 7]. Like human medicine, restenosis frequently complicates procedures like percutaneous coronary interventions in the veterinary practice field [8]. Restenosis in veterinary patients involves intricate pathophysiological mechanisms, similar to those seen in humans [9]. The initial response to vascular injury involves inflammation and the formation of a neointima, composed primarily of smooth muscle cells and an extracellular matrix [10-12]. Over time, this neointima undergoes remodelling, leading to the re-narrowing of the vessel lumen and potentially compromising blood flow [13]. Addressing restenosis in veterinary patients requires a comprehensive understanding of the contributing factors and potential therapeutic agents that could effectively modulate the restenosis process. Resveratrol and quercetin, two natural polyphenolic compounds, have recently garnered substantial scientific interest due to their promising cardiovascular benefits [14-16]. These polyphenols have demonstrated antioxidant, anti-inflammatory, and anti-proliferative properties, which may have implications in mitigating restenosis in an animal model [17-20]. The cardiovascular effects of resveratrol and quercetin in veterinary medicine are of particular interest in the context of restenosis [21, 22] [20]. Studies in animal models have shown that supplementation with these polyphenols can attenuate oxidative stress and inflammation, leading to reduced smooth muscle cell proliferation and neointimal hyperplasia [23-25]. These findings suggest a potential therapeutic role for resveratrol and quercetin in managing restenosis in veterinary patients.

Therefore, this review aims to explore the interplay between restenosis, the therapeutic potential of resveratrol and quercetin, and the intriguing insights from the "French Paradox" [26-28]. By bridging the translational gap between animal models and human trials, novel and effective therapeutic strategies can be developed to manage restenosis in a comprehensive and targeted manner. Exploring the mechanistic pathways through which resveratrol and quercetin operate in the context of restenosis, encompassing both animal models and human medicine, holds significant promise in uncovering novel treatment modalities that could lead to improved outcomes for both veterinary and human patients.

2. Mechanisms of Restenosis in Human and Veterinary Medicine

Restenosis, the re-narrowing of blood vessels after vascular interventions, poses a significant challenge in both human and veterinary medicine. Despite advancements in treatment approaches, restenosis remains a common complication, warranting a deeper understanding of its underlying mechanisms. In both human and veterinary medicine, restenosis primarily occurs as a response to vascular injury resulting from procedures like percutaneous coronary interventions in humans [29], in-stent percutaneous revascularization of peripheral artery disease [30-32] and coronary artery stenting in companion animals [4, 5, 33, 34]. The initial phase of restenosis involves inflammation and the formation of a neointima, characterized by the proliferation of smooth muscle cells and deposition of the extracellular matrix (Figure 1) [35] [61]. This neointima eventually undergoes remodelling, leading to the re-narrowing of the vessel lumen and potential compromise of blood flow [13]. The mechanisms driving restenosis are complex and multifactorial. Inflammation plays a pivotal role in the initiation and progression of restenosis [13]. Following vascular injury, endothelial cells are disrupted, and platelets are activated, releasing growth factors and cytokines that trigger smooth muscle cell migration and proliferation [36]. The recruitment of inflammatory cells, such as macrophages and T lymphocytes, further contributes to the formation of the neointima [36]. In response to multiple growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-), excessive smooth muscle cell proliferation is recognised as neointimal hyperplasia, a hallmark of restenosis [37]. Additionally, the proliferation and migration of smooth muscle cells are promoted by oxidative stress and reactive oxygen species (ROS) generated immediately following vascular damage [38]. Restenosis is remarkably comparable to its human analogous in veterinary medicine. Studies in animal models have demonstrated comparable mechanisms involving inflammation, smooth muscle cell proliferation, and extracellular matrix deposition [4]. For instance, coronary artery stenting in dogs can lead to restenosis, with neointimal hyperplasia being a predominant factor [33, 37]. Understanding the intricate mechanisms of restenosis in both human and veterinary patients is crucial for the development of targeted therapeutic strategies. By elucidating the key pathways involved in restenosis, researchers can explore novel approaches to mitigate neointimal hyperplasia and promote long-term vessel patency after vascular interventions.

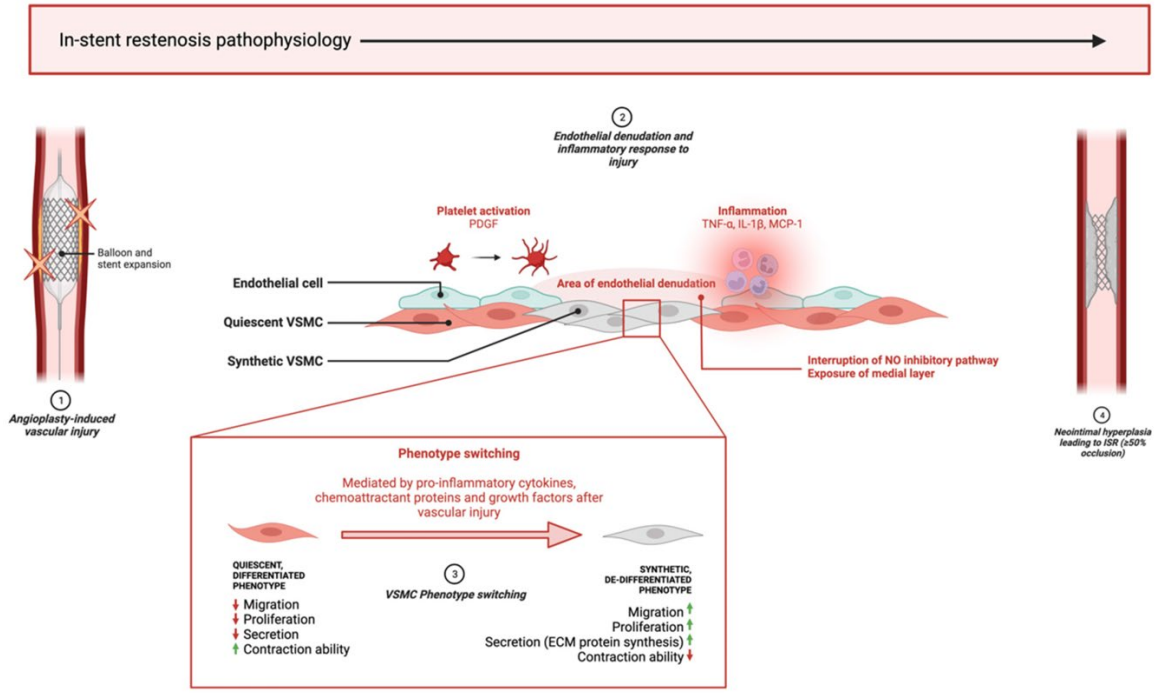

Figure 1. Core Pathological Mechanisms of In-Stent Restenosis. Balloon expansion and stent placement cause mechanical stress, leading to vascular injury, endothelial loss, and inflammation. This prompts inflammatory cells and platelets to release pro-inflammatory factors, initiating a series of events culminating in restenosis. The absence of endothelial protection exposes dormant smooth muscle cells, prompting them to over-proliferate and migrate, contributing to neointima formation. In-stent restenosis occurs when neointimal growth leads to significant lumen narrowing [61]

While the fundamental process of restenosis involves similar cellular and molecular events, there are several factors that can influence the occurrence and progression of restenosis, and these factors can vary between species. Some of the differences include:

a. Vascular Anatomy and Physiology: The structure and physiology of blood vessels can vary between species. Differences in vessel size, wall thickness, and composition can impact the response to injury and the formation of neointima.

b. Cellular Response: The response of smooth muscle cells, endothelial cells, and inflammatory cells to vascular injury can differ between humans and animals. These differences can affect the rate and extent of neointimal growth.

c. Metabolism and Healing: Metabolic rates and healing processes can vary among species. The rate of cell proliferation and migration, as well as the regulation of inflammation and tissue repair, can influence the development of restenosis.

d. Drug Responses: Interventions to prevent restenosis, such as drug-eluting stents, can have varying effects in different species due to differences in drug metabolism, drug delivery, and tissue reactions.

e. Genetic Variation: Genetic factors play a role in susceptibility to restenosis. Genetic variations between species can impact the likelihood and severity of restenosis.

f. Experimental Models: Animal models used to study restenosis may not perfectly replicate the human condition.

Differences in the choice of animal models, such as rodents, rabbits, or pigs, can lead to variations in observed restenosis mechanisms and outcomes. Researchers studying restenosis often use animal models to understand the underlying mechanisms and to test potential therapeutic interventions. While animal models provide valuable insights, it's important to recognize that there may be differences between animals and humans in terms of the exact progression and regulation of restenosis. When extrapolating findings from animal studies to human patients, researchers need to consider these species-specific differences and conduct further studies to validate the findings in the clinical context.

3. Cardiovascular Effects of Quercetin: Mechanisms and Implications for Health

Restenosis is a vexing challenge in both veterinary and human medicine. Despite advances in treatment modalities, the high prevalence of restenosis necessitates further research to develop effective therapeutic strategies. In this context, quercetin, a naturally occurring flavonoid abundantly found in fruits, vegetables, and herbs, has emerged as a promising candidate for managing restenosis due to its potential cardiovascular benefits. This review aims to delve into the multifaceted cardiovascular effects of quercetin and explore the intricate mechanisms underpinning its influence on restenosis in both animal models and human patients. Quercetin exhibits a remarkable impact on the cardiovascular system, making it a focal point in managing restenosis [38]. Its potent antioxidant properties play a pivotal role in scavenging reactive oxygen species (ROS) in vascular tissues, thus reducing oxidative stress, and preserving endothelial cell integrity [39]. The protection of endothelial function is paramount in preventing endothelial dysfunction, a key factor associated with restenosis. Additionally, quercetin's anti-inflammatory effects are essential in suppressing pro-inflammatory cytokines and inhibiting inflammatory signalling pathways [40]. These actions effectively reduce vascular inflammation and mitigate endothelial activation, thus mitigating the risk of atherosclerosis and restenosis [41].

3.1 Mechanisms of Action on Restenosis.

3.1.1 Inhibition of Vascular Smooth Muscle Cell (VSMC) Proliferation and Migration

Quercetin has been shown to effectively inhibit the proliferation and migration of VSMCs, which are key processes in the development of restenosis. Upon vascular injury, VSMCs undergo a phenotypic switch from a contractile to a synthetic phenotype, leading to excessive proliferation and migration, resulting in neointimal hyperplasia and subsequent vessel re-narrowing [42]. The ability of quercetin to alter essential regulatory proteins including cyclin-dependent kinases (CDKs), plays a pivotal role in arresting the cell cycle of VSMCs, thereby reducing their proliferation [40]. Furthermore, quercetin inhibits the expression and activity of matrix metalloproteinases (MMPs), which are responsible for the breakdown of extracellular matrix, preventing VSMC migration and neointimal formation [40].

3.1.2 Promotion of Endothelial Nitric Oxide (NO) Production

Endothelial dysfunction is a critical component of restenosis development, as it compromises vascular integrity and function. Quercetin enhances the production of endothelial nitric oxide (NO), a potent vasodilator, by upregulating endothelial nitric oxide synthase (eNOS) expression [43]. Increased NO bioavailability contributes to improved vasodilation, reduced inflammation, and maintenance of endothelial homeostasis, promoting healthy vascular function and mitigating the risk of restenosis [44].

3.1.3 Anti-Inflammatory and Antioxidant Actions

Quercetin's anti-inflammatory and antioxidant properties also play crucial roles in mitigating restenosis. Inflammation is a significant driver of restenosis, and quercetin's ability to inhibit pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-alpha), helps dampen the inflammatory response within the vessel wall [6]. Additionally, quercetin acts as a potent scavenger of reactive oxygen species (ROS), reducing oxidative stress and preserving the structural and functional integrity of vascular tissues [45]. By curbing inflammation and oxidative stress, quercetin safeguards against endothelial damage and VSMC proliferation, further contributing to restenosis prevention.

3.1.4 Modulation of Signalling Pathways

Quercetin's regulatory effects extend to several signalling pathways implicated in restenosis pathogenesis. Notably, it has been found to suppress the mitogen-activated protein kinase (MAPK) signalling pathway, which is crucial for VSMC proliferation [14]. Additionally, quercetin inhibits the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathway, which promotes VSMC survival and migration [43]. By targeting these signalling pathways, quercetin effectively hampers VSMC proliferation and migration, contributing to restenosis prevention.

3.2 Cardiovascular Effects of Quercetin: Insights from Multiple Studies

Several noteworthy studies have explored quercetin's potential therapeutic effects on restenosis. In a rat carotid artery balloon injury model, Huang et al. (2009) investigated quercetin's ability to attenuate restenosis. The study demonstrated that quercetin treatment significantly reduced neointimal formation and VSMC proliferation, shedding light on its potential for managing restenosis [46]. Similarly, Thipparaboina et al. (2003) evaluated quercetin's protective effects against restenosis in a porcine coronary artery stent model. Their findings showcased that quercetin treatment effectively inhibited inflammatory responses and reduced neointimal hyperplasia, underscoring its anti-inflammatory and anti-proliferative properties in the context of restenosis [47]. Dagner et al. (2014) conducted a study to examine quercetin's protective effects against radiation-induced endothelial cell apoptosis. Human umbilical vein endothelial cells were exposed to radiation, and quercetin was administered to assess its impact. The results indicated that quercetin protected endothelial cells against apoptosis through the PI3K/Akt pathway, implying its potential role in mitigating endothelial damage associated with restenosis [48]. Cheng et al. (2019) Cheng et al. explored quercetin's anti-inflammatory effects in ARPE-19 cells. The study revealed that quercetin effectively inhibited IL-1beta-induced production of inflammatory cytokines and chemokines through the MAPK and NF-kappa signalling pathways. These findings suggest that quercetin's anti-inflammatory properties may be beneficial in reducing vascular inflammation associated with restenosis [49]. Moon et al. (2016) Moon et al. conducted a study to investigate the effect of quercetin on intimal hyperplasia in a rat carotid artery balloon injury model. Quercetin treatment significantly reduced neointimal hyperplasia and smooth muscle cell proliferation, indicating its potential as a restenosis-inhibiting agent [40]. These studies further support the notion that quercetin holds promise as a therapeutic agent in managing restenosis. The diverse range of research conducted in various animal models highlights quercetin's potential benefits in inhibiting neointimal hyperplasia, reducing smooth muscle cell proliferation, and suppressing vascular inflammation. Although more clinical trials are warranted to validate these findings in human patients, the evidence

from these studies emphasizes quercetin's potential significance as a restenosis-targeting agent in both veterinary and human medicine.

4. Cardiovascular Effects of Resveratrol: Mechanisms and Implications for Health

Resveratrol exhibits remarkable effects on the cardiovascular system, making it a focal point in managing restenosis. Its potent antioxidant properties play a crucial role in scavenging reactive oxygen species (ROS) in vascular tissues, thereby reducing oxidative stress, and preserving endothelial cell integrity [50]. The preservation of endothelial function is vital in preventing endothelial dysfunction, a key factor associated with restenosis. Moreover, resveratrol's anti-inflammatory effects are pivotal in suppressing pro-inflammatory cytokines and inhibiting inflammatory signalling pathways [51]. These actions effectively mitigate vascular inflammation and reduce endothelial activation, thereby lowering the risk of atherosclerosis and restenosis.

4.1 Mechanisms of Action on Restenosis

4.1.2 Inhibition of Vascular Smooth Muscle Cell (VSMC) Proliferation and Migration

Resveratrol exerts a potent inhibitory effect on VSMC proliferation and migration, which are key processes contributing to neointimal hyperplasia and restenosis. Studies have demonstrated that resveratrol downregulates the expression of cyclin-dependent kinases (CDKs) and matrix metalloproteinases (MMPs) in VSMCs, leading to a reduction in their proliferation and migration [19, 52]. By modulating these critical regulatory proteins, resveratrol effectively hinders the excessive growth and movement of VSMCs, ultimately preventing the re-narrowing of blood vessels [52].

4.1.3 Antioxidant Properties and Reduction of Oxidative Stress

Resveratrol's potent antioxidant properties play a pivotal role in reducing oxidative stress within vascular tissues. By scavenging reactive oxygen species (ROS) and neutralizing free radicals, resveratrol protects endothelial cells from damage and preserves their function [53]. This antioxidative action is crucial in maintaining endothelial health and preventing endothelial dysfunction, a key factor associated with restenosis [53].

4.1.4 Anti-Inflammatory Effects and Suppression of Pro-Inflammatory Cytokines

The pathogenesis of restenosis includes inflammation significantly. Tumour necrosis factor-alpha (TNF-alpha) and interleukins are two pro-inflammatory cytokines that have been demonstrated to be suppressed by resveratrol, which lowers vascular inflammation [54]. Resveratrol reduces endothelial activation and the infiltration of immune cells into the vascular wall by inhibiting inflammatory signalling pathways, consequently lowering the risk of restenosis [55].

4.1.5 Activation of Endothelial Nitric Oxide (NO) Production

Endothelial Nitric Oxide (NO) production is critical for maintaining vascular homeostasis and function. Resveratrol has been found to promote endothelial NO production, leading to enhanced vasodilation and improved vascular endothelial function [54]. The increased bioavailability of NO supports optimal vascular tone and blood flow, contributing to overall cardiovascular health and reducing the likelihood of restenosis [55].

4.2. Cardiovascular Effects of Resveratrol: Insights from Multiple Studies

Several noteworthy studies have explored resveratrol's potential therapeutic effects on restenosis. In a randomized clinical trial by Diaz et al. (2009), patients undergoing percutaneous coronary intervention (PCI) were administered resveratrol or placebo. The resveratrol group exhibited a significant reduction in restenosis rates and improved vascular function compared to the placebo group, indicating its potential as an adjunct therapy in PCI [56]. A study by Li et al. (2018), The article explores the potential of resveratrol in reducing oxidative stress induced by balloon injury in the rat carotid artery. It focuses on the mechanisms of action involving the ERK1/2 and NF-kappa B pathways. The study demonstrates that resveratrol effectively attenuates oxidative stress, which plays a crucial role in the pathogenesis of restenosis. These findings suggest that resveratrol may hold promise as a therapeutic agent for managing restenosis by targeting specific cellular signalling pathways associated with oxidative stress. A study by Zhang et al 2013 explores the potential of resveratrol in reducing oxidative stress induced by balloon injury in the rat carotid artery. It focuses on the mechanisms of action involving the ERK1/2 and NF-kappa B pathways. The study demonstrates that resveratrol effectively attenuates oxidative stress, which plays a crucial role in the pathogenesis of restenosis. These findings suggest that resveratrol may hold promise as a therapeutic agent for managing restenosis by targeting specific cellular signaling pathways associated with oxidative stress. Elmadhun et al. 2013, discuss the potential of pigs as a valuable animal model for studying the effects of resveratrol in preventing cardiovascular disease. The researchers emphasize the importance of using pigs due to their physiological similarities to humans, especially in terms of cardiovascular anatomy and function. The study highlights the cardiovascular benefits of resveratrol, a natural polyphenolic compound, and its potential to mitigate cardiovascular diseases, including restenosis. The authors explore the mechanisms of action of resveratrol in pigs, such as its antioxidant and anti-inflammatory properties, which may contribute to improved cardiovascular health. The findings suggest that using pigs as a model for resveratrol research could provide valuable insights for developing effective therapeutic strategies for cardiovascular diseases in both veterinary and human medicine Fields [57]. 이미희, 2012 explores using bioactive compounds to prevent intimal hyperplasia in small-caliber vascular grafts, aiming to improve graft patency and long-term outcomes in vascular surgeries. These compounds, like growth factors and cytokines, can modulate cellular responses and promote a more favourable healing process, reducing inflammation and smooth muscle cell proliferation [58]. According to Gu et al.2006, the study investigates the effects of resveratrol on endothelial progenitor cells (EPCs) and their role in reendothelialization in rats with intima injury. Resveratrol treatment was found to enhance EPC function and promote their incorporation into the injured intima, contributing to improved reendothelialization. These findings suggest that resveratrol may have potential therapeutic benefits in promoting vascular healing and repair [59]. These preclinical studies provide valuable evidence supporting the potential therapeutic effects of resveratrol on restenosis in various animal models. However, it is essential to interpret these findings with caution, as results from animal studies may not directly translate to human clinical outcomes. Further research, including well-designed clinical trials, is necessary to validate the safety and efficacy of resveratrol in managing restenosis in humans.

Table 1. Summary of Mechanisms of Action and Cardiovascular Effects of Quercetin and Resveratrol in Restenosis.

Mechanism/Effect	Quercetin	Resveratrol
Inhibition of VSMC Proliferation and Migration	- Alters cyclin-dependent kinases (CDKs) to arrest the VSMC cell cycle, reducing proliferation - Inhibits matrix metalloproteinases (MMPs) to prevent VSMC migration	- Downregulates CDKs and MMPs in VSMCs, suppressing proliferation and migration - Suppresses VSMC proliferation and migration by modulating key proteins
Promotion of Endothelial NO Production	- Upregulates endothelial nitric oxide synthase (eNOS) expression, enhancing NO production - Increases nitric oxide (NO) bioavailability for healthier vascular tone	- Promotes endothelial NO production, improving vasodilation and function - Enhances vascular endothelial function, contributing to reduced restenosis risk
Anti-Inflammatory and Antioxidant Actions	- Inhibits pro-inflammatory cytokines, such as IL-6 and TNF-alpha - Acts as a potent scavenger of reactive oxygen species (ROS), reducing oxidative stress	- Suppresses pro-inflammatory cytokines, reducing vascular inflammation - Scavenges ROS, mitigating oxidative stress and preserving endothelial integrity
Modulation of Signalling Pathways	- Suppresses mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways	- Modulates ERK1/2 and NF-kappa B pathways, influencing VSMC proliferation and survival
Cardiovascular Effects	- Reduces neointimal hyperplasia through inhibition of VSMC growth - Enhances vascular NO levels, improving vasodilation and overall function - Mitigates inflammation and oxidative stress, protecting vascular tissues	- Reduces neointimal hyperplasia, limiting vessel re-narrowing - Enhances endothelial NO production, contributing to healthy vascular tone - Suppresses inflammation and oxidative stress, promoting cardiovascular health
Additional Mechanisms	- Alters essential regulatory proteins, arresting VSMC cell cycle - Inhibits MMPs, preventing neointimal formation - Upregulates eNOS expression, increasing NO production - Modulates signalling pathways related to VSMC proliferation and migration	- Antioxidant properties scavenge ROS, protecting endothelial cells - Anti-inflammatory effects reduce endothelial activation - Potential benefits in small-caliber grafts for restenosis prevention

Disclaimer: The following table presents a summary of the mechanisms of action discussed in the provided review.

5. Discussions

The comprehensive review of resveratrol and quercetin's cardiovascular effects and their potential in managing restenosis reveals promising therapeutic implications for both veterinary and human medicine. These natural polyphenolic compounds have demonstrated antioxidant, anti-inflammatory, and anti-proliferative properties, which play pivotal roles in preserving endothelial function, mitigating vascular inflammation, and inhibiting VSMC proliferation and migration. The mechanisms of action underlying their effects on restenosis involve modulation of key regulatory proteins, suppression of inflammatory signalling pathways, and promotion of endothelial NO production. Restenosis is a complex and multifactorial process that occurs in response to vascular injury, particularly after interventions such as angioplasty or stent placement. The primary goal of these procedures is to open narrowed or blocked blood vessels and restore blood flow. However, the healing process that follows can lead to excessive tissue growth within the vessel, resulting in restenosis and re-narrowing of the blood vessel. Resveratrol and quercetin's inhibitory effects on VSMC proliferation and migration hold promise for preventing neointimal formation and restenosis after vascular interventions. Additionally, their ability to reduce vascular inflammation and oxidative stress can contribute to preserving endothelial health and preventing endothelial dysfunction, crucial factors in restenosis management. The intriguing "French Paradox" further highlights the potential cardiovascular benefits of polyphenols, including resveratrol, found in red wine and polyphenol-rich foods. The observation of a low incidence of heart disease in the Southern French population despite a diet rich in saturated fats and cholesterol has piqued interest in exploring the effects of polyphenols on cardiovascular health, including their role in restenosis. The cardiovascular effects of resveratrol and quercetin observed in animal models suggest that their therapeutic potential might extend to both veterinary and human medicine. Clinical studies evaluating the effects of resveratrol and quercetin on restenosis in humans have also provided promising findings. Randomized clinical trials in patients undergoing percutaneous coronary intervention (PCI) have shown that resveratrol administration is associated with reduced restenosis rates and improved vascular function compared to placebo [60]. Despite the promising preclinical evidence, several challenges must be addressed before implementing resveratrol and quercetin as restenosis management strategies in veterinary and human medicine. One significant hurdle is the translational gap between animal models and human clinical trials. While animal studies provide essential insights into their mechanisms of action and safety, human trials are necessary to validate their efficacy and potential side effects in real-world scenarios. Moreover, the optimal dosing and formulation of resveratrol and quercetin for restenosis management need to be determined, ensuring maximum effectiveness while minimizing potential adverse effects. The investigation of a new drug delivery system presents a promising avenue in the context of restenosis management, specifically targeting the therapeutic potential of resveratrol and quercetin. A novel drug delivery approach seeks to overcome challenges related to the limited bioavailability, rapid degradation, and clearance of polyphenolic compounds, which can hinder their effectiveness in mitigating restenosis. By encapsulating resveratrol and quercetin within biocompatible carriers, such as nanoparticles or microparticles, this new drug delivery system enables precise and controlled release of the active substances at the site of injury. The localized delivery mechanism holds the potential for sustained release over an extended period following stent implantation, thereby providing a prolonged therapeutic effect during the critical phase of vascular healing and restenosis prevention. The advantages of this approach lie in its ability to optimize the concentration of active compounds at the injury site, enhancing their therapeutic efficacy and reducing the risk of off-target

effects or systemic toxicity. Furthermore, the controlled release kinetics ensure a continuous and targeted intervention, offering a more comprehensive and sustained response to restenosis. Despite the promising outlook, the successful translation of the new drug delivery system into clinical practice necessitates rigorous research and development. Key areas of focus include optimizing the carrier's biocompatibility, stability, and release profiles, as well as conducting thorough safety and efficacy assessments in preclinical and clinical settings. Such advancements in drug delivery technology hold substantial implications for the field of cardiovascular medicine. By leveraging the potential of this innovative approach, researchers and clinicians can improve restenosis management in both human and veterinary patients. However, challenges remain, such as ensuring regulatory compliance and addressing potential side effects, which require meticulous attention and further investigation.

6. Conclusion

In conclusion, the investigation of resveratrol and quercetin in the context of restenosis presents promising prospects for both human and veterinary medicine. Their antioxidant, anti-inflammatory, and anti-proliferative properties make them potential candidates for developing innovative therapeutic strategies. The exploration of a new drug delivery system offers a promising solution to enhance the therapeutic application of resveratrol and quercetin in restenosis management. The localized and sustained release of these polyphenolic compounds may revolutionize the approach to cardiovascular interventions, leading to more effective and targeted treatments for restenosis and ultimately improving patient outcomes in both veterinary and human medicine. As research progresses, a novel drug delivery system could become a transformative tool in the fight against restenosis, bridging the gap between scientific exploration and clinical application in the realm of cardiovascular health.

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Review

Flow cytometry and its use in modern human and veterinary andrology

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Abstract: Fertility of a male is sometimes difficult to assess and results obtained using regular sperm analysis methods are often inconclusive. Flow cytometry was proven to generate crucial information, that allows specialists to conclude upon the reproductive capacity of an individual. Together with computer assisted sperm analysis CASA systems, flow cytometers allow an in-depth analysis of fresh, chilled or frozen/thawed semen, which is currently essential for research purposes, but also for diagnosis of various male-related fertility disorders, both in veterinary and human medicine. Several parameters can be assessed using this method, such as sperm viability, acrosome integrity, mitochondrial activity, concentration and total sperm number, DNA fragmentation, capacitation, oxidative stress, etc. This paper provides a rapid reference to specialists involved in semen analysis by flow cytometry, regarding semen sample preparation, instrument setup, and evaluation of the most important sperm parameters (viability, acrosome reaction and mitochondrial activity).

Keywords: flow cytometry; mammalian sperm; viability; acrosome integrity; mitochondrial activity.

1. Introduction

Flow cytometers are made up of several independent systems that are interconnected to yield the final results, such as fluidics, that allows a single-stranded alignment of events, optics, made up of several lasers, light filters and detectors as well as electronics, which allows conversion of optical signals into electronic information which can be stored and analysed by a computer, equipped with a dedicated software. As such, flow cytometry represents a powerful technique that allows a complex and prompt evaluation, or even separation, of single cells (called events) found in suspension, making it therefore very suitable for sperm analysis. Cells can be thus evaluated regarding their size (forward scatter), internal complexity usually given by their granularity (side scatter) as well as fluorescent intensity (when stained with fluorescent antibodies or dyes that bind to the nucleus, cytoplasm, or membrane) [1]. Since fertility of a male is sometimes difficult to assess and results obtained using regular sperm analysis methods are often inconclusive, flow cytometry was proven to generate crucial information, that allows specialists to conclude upon the reproductive capacity of an individual. Several parameters can be assessed using this method, such as sperm viability, acrosome integrity, mitochondrial activity, concentration and total sperm number, DNA fragmentation, capacitation, oxidative stress, etc. [2]. This paper is aimed at providing a rapid reference to specialists involved in semen analysis by flow cytometry, regarding semen sample preparation, instrument setup, and evaluation of the most important sperm parameters (viability, acrosome reaction and mitochondrial activity).

2. Semen Sample Preparation

Semen analyzed by flow cytometry may originate from fresh ejaculates, collected by any of the commonly used methods (masturbation, artificial vagina, electroejaculation,

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etc.) but may also be obtained after flushing of epididymis (from dead/slaughtered animals or following castration). Frozen semen samples may also be investigated by flow cytometry and such studies are of particular interest to assess efficacy of the freezing method or suitability of the extender used [3].

One of the biggest issues that has to be taken into consideration is interference between components of the seminal plasma or extender and staining dyes. In cases where this represents a serious concern, the most suitable method of sample clean-up or washing has to be employed. Usually, a density gradient centrifugation or the swim-up technique is adequate.

Another significant problem is related to the debris which is often present in the sperm extenders, due to the egg yolk or milk proteins. If debris is not removed or gated out, it can lead to false results as foreign particles may overlap unstained populations of spermatozoa [4]. On the other hand, there are dyes such as tetraethylbenzimidazolylcarbocyanine iodide (JC-1) which are lipophilic and therefore can bind to debris and yield misleading results. Gating out debris can be achieved on the FSC vs SSC dot plot, where it usually appears to have significantly lower FSC as spermatozoa.

Mathematical corrections are also possible but are time consuming and difficult to perform.

Another option is to use an intravital dye, such as Hoechst 33342 which only stains live cells, and therefore unstained events can be gated out as debris. The advantage of using this dye also resides in the fact that its fluorescent signal (UV range) does not overlap with any of the fluorochromes frequently used for semen analysis [5]. Nevertheless, the drawback is that the cytometer must have a violet laser or UV diode.

3. Instrument setup

In order to obtain accurate results, the flow cytometer must be checked and properly calibrated on a daily basis. Calibration microspheres are usually made of polystyrene latex and are labelled with fluorescent dyes. Each laboratory must establish a daily clean-up and calibration protocol, according to the type of equipment that is present and the instructions of the producer. Standardization of the procedure is very important, as it provides reproducibility, accuracy and reliability of results.

Next, the machine can be prepared for analysis, by choosing the optimum channels and optical filters needed, according to the type of experiment that is required. Usually, the blue 488 nm Argon ion laser at 488 nm is the only one that is needed, since most of the dyes used for sperm analysis emit green, orange or red fluorescence. Green fluorescence is read in FL1, orange in FL2 and red in FL3. Regarding filters, the 530/28 BP should be used for FL1, the 585/42 BP for FL2 and the 650 LP for FL3. All dot plots or histograms should be set to the logarithmic scale and signal height should be acquired for all parameters.

Compensation should always be performed, whenever multiple dyes are used to stain the same semen sample, in order to avoid fluorescence spill over and false results. This is usually achieved using compensation beads, that are stained with the same fluorochromes as those used in the experiment. Experienced users may also choose to perform manual compensation, after the samples are acquired.

Actual analysis begins by plotting forward scatter height (FSC-H) vs side scatter height (SSC-H) in order to define and gate the sperm population. This will also allow to remove from the gate any debris (mostly originating from the extenders) and also to eliminate any electronic noise. A total number of at least 10,000 events should be acquired, and, since the cells (spermatozoa) are small, the sample should be run at low speed for better accuracy.

4. Sperm viability assessment

DNA intercalating agents are fluorescent molecules, capable of passing through cellular membranes of cells and therefore stain the nuclei. Some of the molecules are able to pass through intact membranes and therefore also stain the nuclei of live cells. A frequently utilized green fluorescent dye, that stains all nuclei of spermatozoa (damaged or intact), is SYBR-14 (maximum emission at 516 nm) which is used in combination with a red dye - propidium iodide (PI, maximum emission at 617 nm). The latter is also an intercalating agent which can only penetrate the damaged plasma membrane and therefore stains the nucleus of only dead spermatozoa. PI signal quenches SYBR-14 fluorescence, thus live spermatozoa are stained in green while dead spermatozoa are red [6]. Nowadays, there are several live/dead kits available on the marked, which allow differentiation between live and dead spermatozoa. Those kits also permit an easy discrimination between spermatozoa and debris, and therefore eliminate the need of performing additional staining steps.

These kits usually contain a 1 mM solution of SYBR-14 in DMSO and a 2.4 mM solution of PI in water. A 20 μM SYBR-14 stock solution in DMSO is initially prepared, which can be stored frozen. When needed, the working solution is made by adding 5 μL of the 20 μM SYBR-14 solution and 50 μL of the 2.4 mM PI solution to 10 ml of buffer, such as HEPES for approximately 20 samples.

Semen samples can be successfully stained with this working solution, although the manufacturers recommend separate incubations. Spermatozoa should be diluted to a concentration of 1-2x106/ml in 0.5 ml staining solution, in cytometry tubes. Incubation should be made in the dark at 37°C and run in the cytometer right away. Two dot plots are needed for each tube, one for spermatozoa gating (FSC-H vs SSC-H) and another one for viability assessment (FL1-H vs FL3-H). Following the analysis, 3 distinct populations are visible on the dot plot:

- SYBR-14+/PI– (live spermatozoa);
- SYBR-14+/PI+ (moribund spermatozoa);
- SYBR-14–/PI+ (dead spermatozoa).

The SYBR-14–/PI– events are likely to represent debris which should be gated out.

Alternatively, sperm viability can be assessed using a combination of 3 fluorescent dyes - SNARF-1, YO-PRO-1 and ethidium homodimer. Thus, 4 subpopulations of spermatozoa can be detected: one viable, with stable membranes (SNARF-1+), and three with compromised membranes: YO-PRO-1+/Eth-, YO-PRO-1-/Eth+ and YO-PRO-1+/Eth+ [7,8].

5. Evaluation of acrosome reaction

The acrosome is a structure that covers the anterior part of spermatozoa in mammals and contains enzymes that allow penetration of the zona pellucida during fertilization. Semen cryopreservation sometimes induces a so-called acrosome reaction, which means inactivation of the specific acrosomal enzymes, which renders spermatozoa inefficient [9]. Acrosome integrity can be assessed using FITC or PE labelled plant lectins (pea agglutinin-PSA or peanut agglutinin-PNA). PSA cannot breach the membrane of an intact acrosome and thus, if stained, spermatozoa are judged as damaged. In practice, PNA is preferred to PSA as the latter was demonstrated to non-specifically bind to the egg-yolk found in semen extenders as well as to other fragments of spermatozoa [10].

To prepare the stock solution, lyophilized PNA-FITC is resuspended in water to a concentration of 0.1-1 mg/ml and then stored frozen until use. The 1 mg/ml solution is usually preferred. If storage at the refrigeration temperature is needed, a supplementation with 2 mM Na-azide is mandatory.

The usual protocol when assessing the acrosome reaction is to combine PNA-FITC staining with PI, in order to also observe the population of non-viable spermatozoa. The PI stock solution can also be stored frozen, at a concentration between 50 μg-5mg/ml, but most frequently a 1 mg/ml solution is preferred.

The working solutions can easily be prepared based on the stock solutions at 1 mg/ml. After thawing, 10 μl of both the PNA-FITC and PI solutions are added to 10 ml of PBS and 0.5 ml of the resulting solution are used to dilute the semen sample to a concentration of 1-2 x 106 spermatozoa/ml. The working solution should be kept in the darkness until use, but not more than 12-24 hours. After staining, spermatozoa should be incubated in darkness for 15 minutes at 37°C.

Next, the samples are run in the flow cytometer and two dot plots are needed: one for spermatozoa gating (FSC-H vs SSC-H) and another for acrosome reaction/viability (FL1-H vs FL3-H).

Following analysis, four different populations of spermatozoa can be identified:

- the PNA-FITC–/PI– population is represented by viable spermatozoa with unreacted acrosome;
- the PNA-FITC+/PI– population is represented by viable spermatozoa with reacted acrosome;
- the PNA-FITC+/PI+ population is represented by moribund or dead spermatozoa with reacted acrosome;
- the PNA-FITC–/PI+ population is represented by moribund or dead spermatozoa with unreacted acrosome.

6. Evaluation of mitochondrial activity

In mammalian spermatozoa, mitochondria are essential organelles which play a crucial role for their motility and fertilizing capability, by contributing to ATP and reactive oxygen species production as well as calcium level control. Their integrity and activity can be assessed by quantitatively evaluating their

membrane potential, using a fluorescent dye called 5,5,6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimi-dazoylcarbocyanine iodide (JC-1). When mitochondrial membrane potential is high, JC-1 forms combinations that produce red fluorescence, while in the case of low mitochondrial membrane potential, JC-1 stays monomeric and emits green fluorescence (11).

The JC-1 stock solution can be prepared by resuspending the lyophilized powder in DMSO, to a concentration of 2 mg/ml (3 mM) and stored frozen in a dark vial. The working solution is a 1000 fold dilution of the stock solution. Therefore, 10 μl of the stock solution should be added to 10 ml of PBS and 0.5 ml of the resulting solution are used to dilute the semen sample to a concentration of 1-2 x 106 spermatozoa/ml. Following incubation for 15-20 minutes in darkness at 37°C, samples are run in the flow cytometer and visualized in two dot plots: one for gating the spermatozoa (the usual FSC-H vs SSC-H) and another for mitochondrial membrane potential (FL1-H vs FL2-H).

Analysis of dot plots allows classification of ejaculates according to mitochondrial activity of spermatozoa, as follows:

- spermatozoa with high FL1-H and low FL2-H have a low mitochondrial membrane potential and are considered of low fertilizing ability;
- spermatozoa with low FL1-H and high FL2-H have high mitochondrial membrane potential and potentially poses a fertilizing ability (this category should be the most abundant in good quality ejaculates);
- spermatozoa with high FL1-H and high or moderate FL2-H are considered to have large gaps in their mitochondria and are therefore of lower quality;
- spermatozoa with low FL1-H and low FL2-H are likely dead and have a damaged midpiece.

7. Conclusions

Flow cytometry is an extremely useful and powerful tool that can be used for advanced semen analysis as it provides quick and reliable results, enabling an accurate estimation of various semen parameters.

Together with computer assisted sperm analysis CASA systems, flow cytometers allow an in-depth analysis of fresh, chilled or frozen/thawed semen, which is currently essential for research purposes, but also for diagnosis of various male-related fertility disorders, both in veterinary and human medicine.

The equipment required is indeed quite expensive and the operators need special training, while experience is also an asset.

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