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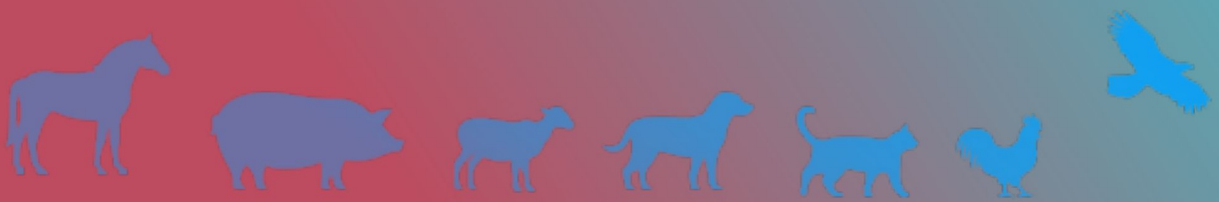

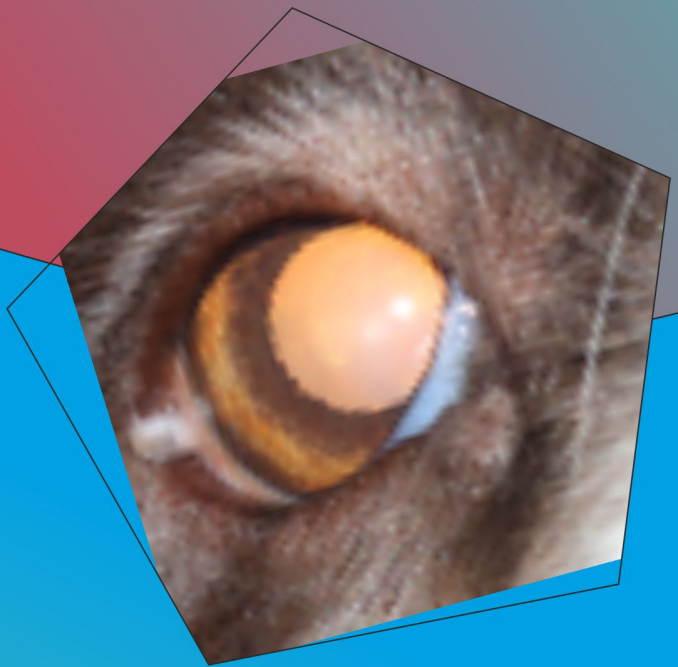

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Communication

Assessing two strategies for production of murine ascites with anti-SARS-CoV-2 monoclonal antibodies

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Abstract: Studies were conducted to improve the production of murine ascites with monoclonal antibodies that recognize SARS-CoV-2 proteins. BALB/c mice were primed with 0.5 mL of mineral oil into the abdominal cavity. Seven days after priming, mice were divided in two groups: the group 1 was inoculated intraperitoneally with $2x10^6$ cells/mL of MAb-secreting hybridomas against the nucleocapsid and spike proteins of SARS-CoV-2; the group 2 was injected simultaneously with the same inoculum of hybridoma cells and mineral oil, 18 days after priming. No disturbances or suffering signals were observed in mice from both groups, suggesting that double administration of mineral oil did not produce significant distress with respect to the single dose used for priming, and that none of the hybridoma cell lines were particularly aggressive for the inoculated mice. Ascites was collected in 90.48% and 97.68% of mice from groups 1 and 2, respectively. Ascites was not collected in 7.42% of all mice. The main cause was they never developed ascites tumors but no solid tumors were observed either. The volume of ascitic fluid per mouse was increased significantly in mice from group 2, and there were no significant differences between groups in terms of the concentration of IgG in clarified ascites. According to these results, to obtain higher amounts of MAb the strategy applied in group 2 should be used, since it showed the best results in the development of ascites tumors and it significantly increased the volume of ascites fluid per mouse. This could allow the use of fewer animals for ascites production, which is an ethical and economic benefit.

Keywords: ascites; hybridoma; mice; mineral oil

1. Introduction

At the end of 2019, a new coronavirus began to spread in China to become the pandemic that has cost the most human lives: the SARS-CoV-2. All efforts of healthcare personnel and scientists have had to focus on its rapid diagnosis and treatment. In the strategies to reduce the viral transmission, monoclonal antibodies (MAbs) have become reliable tools, especially for immunoassay techniques used for diagnosis, which are cost-effective, sensitive, rapid and selective [1].

There are basically two main phases in the production of MAbs: the selection of MAb-producing hybridoma cells, generated by the fusion of antibody-producing lymphoid cells from an immunized mouse and murine myeloma cells, and the propagation of selected hybridoma clones, in *vivo* or in *vitro*. The *in vivo* production of MAbs has been carried out by injecting the hybridoma cells into the mice abdominal cavity. The propagation of these cells in the ascitic fluid offers a rapid and economical route to the production of MAbs [2]. *In vitro* production techniques have been developed over the years as alternatives to *in vivo* production of MAbs, but many of these techniques have not been practical due to their high cost, requirements for specialized equipment or their propensity to become contaminated [3]. MAb production in transgenic plants is also a promising technology, since they are considered inexpensive and facile production platforms for recombinant MAbs [4], but it still not solves the great demand of these molecules. Consequently, the total replacement of the ascites method is not yet possible, much less in the current pandemic context, when the rapid and effective production of MAbs is needed for the diagnosis and control of viral transmission.

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Under these circumstances, medium or large-scale productions of MAbs are required. Medium-scale production is demanded to make 0.1-1.0 g quantities for diagnostic or developing assays [3]. Large-scale production requires an extensive inoculation program with a large number of mice, mostly when a single mouse is inoculated with a single antigen. Hence, from an ethical and economic perception, it would be necessary to acquire efficient and high throughput strategies to maximize the MAb production and to reduce the number of animals used [5].

The Center for Genetic Engineering and Biotechnology of Sancti Spiritus (CIGBSS), Cuba, has been in charge of generating several MAb-secreting hybridomas against the nucleocapsid and spike proteins of SARS-CoV-2 virus. These antibodies must be produced to satisfy the increasing demands of the enzyme-linked immunosorbent assay (ELISA) applied currently for detecting SARS-CoV-2 antigen. The present work aimed to improve the production of murine ascites containing anti-SARS-CoV-2 MAbs, by assessing two strategies for the administration of mineral oil for BALB/c mice: a single injection, as priming agent, 7 days before the inoculation of hybridoma cells and the simultaneous injection of mineral oil and hybridoma cells to mice previously primed with mineral oil. The capability of mice to develop ascitic tumors, the volume of ascites per mouse and the IgG concentration in clarified ascites were monitored to select the best strategy for production of ascites with anti-SARS-CoV-2 MAbs.

2. Materials and Methods

2.1 Hybridoma cell lines

For the production of anti-SARS-CoV-2 MAbs in murine ascites, there were used six hybridoma cell lines generated by CIGBSS, Cuba. Three of them secrete MAbs that recognize the SARS-CoV-2 nucleocapsid protein (CBSSNCOV.2, CBSSNCOV.3 and CBSSNCOV.10) and the others recognize the receptor binding domain (RBD) of the SARS-CoV-2 spike protein (CBSSRBD-S.1, CBSSRBD-S.2 and CBSSRBD-S.3).

2.2 Mouse priming and inoculation

BALB/c male and female mice of 22 ± 1 and 24 ± 1 g of weight, respectively, were used for ascitic fluid production. They were maintained in Eurostandard type II cages (268 mm x 215 mm x 141 mm) at (22-25) °C and 50-65% relative humidity. All animals were primed with 0.5 mL of mineral oil (Zahori, Mexico) in the abdominal cavity. Each hybridoma inoculum was prepared at a dilution of 2 x 10^6 cells per milliliter of RPMI-1640 medium (Sigma, Hybri-Max). Before the inoculation of cells, the mice were divided into two groups: the group 1 was inoculated intraperitoneally with 1 mL of cell suspension seven days after priming, and the group 2 was inoculated analogously to group 1 but 18 days after priming and simultaneously with 0.5 mL of mineral oil.

The mice were observed at least twice daily, including weekends, by personnel familiar with the clinical signs related to the production of ascites, to assess the degree of abdominal distention and to monitor health and well-being.

All the experimental procedures were approved by the Ethical Committee on Animal Experimentation of the Center for Genetic Engineering and Biotechnology (CIGB, Havana, Cuba).

2.3 Ascites harvest

The extraction of ascites was carried out in two times: 10 and 12 days after the inoculation of the hybridoma cells. Before the ascites extraction, the abdomen of each mouse was cleaned with 70% ethanol. The abdominal paracentesis was performed in the right side of the inguinal-abdominal region with an 18-gauge needle, inserted at a 35-45° angle. The ascites fluid was harvested in 50 mL Corning tubes with 150 µL of 8% EDTA. The ascites was clarified by centrifugation at 1125 x g for 30 min at (22-25) °C and filtered through 0.45 µm glass wool.

2.4 Quantification of IgG in clarified ascites

The quantification of murine IgG in clarified ascites was performed by a sandwich ELISA. Polystyrene 96-well microtiter plates (Costar 3590 High Binding) were coated with 10 µg/mL goat anti-mouse IgG polyclonal antibody in 100µL/well coating buffer (10mM carbonate-bicarbonate buffer, pH 9.6) and incubated 2 h at 37°C. Wells were washed twice with 380µL/well washing buffer (0.05% Tween-20) and blocked with 200µL/well blocking buffer (phosphate-buffered saline, pH 7.2, and 1% nonfat milk). This and the two subsequent steps were carried out 1 h at 37°C. The plate was washed once and 100 µL of ascites diluted 1:20 0000 with blocking buffer were added to wells. The calibration curve was prepared in the range of 150 ng/mL to 2.34 ng/mL using the CBSSPSA.4, supplied by CIGBSS as standard MAb. Plates were washed three times and 100µL of peroxidase

(HRP)-conjugated goat anti-mouse IgG diluted 1:8 000 with blocking buffer were added per well and incubated. After four washes, 100µL/well 5.5mg/mL o-phenylenediamine dihydrochloride with 0.015% hydrogen peroxide in 0.1M citrate-phosphate buffer, pH 5.0, were added and incubated 20 min at (22-25) °C in the dark. The reaction was stopped with 100µL/well of 2M sulfuric acid, and the absorbance was measured at 492 nm in a microplate reader (Labsystems Multiskan® Plus, Finland).

2.5 Statistical analysis

The data of ascites volume per mouse and IgG concentration in clarified ascites, obtained from each group of mice, were analyzed by means of independent samples t-test (α=0.05) using the Statistical Package for Social Science, version 15.0.

3. Results

Mice developed the gradual swelling of the abdomen that accompanies the accumulation of ascites fluid over a period of 7 days after the injection of hybridoma cells. The daily observation did not detect evidence of distress in the animals of both groups under study: their coats were clean and smooth, they maintained food and water consumption and the activity into the cages was normal in all of them.

The first extraction of ascites was performed 10 days after the inoculation of the cells. At that time, the abdominal distention was moderate since ascites fluid volumes did not exceed 20% of the baseline body weight of mice. Each mouse was tapped two times. After the second and last tap, the following clinical irregularities were perceived in some animals of both groups: hunched posture, piloerection and decreased activity.

Table 1 shows the number of mice used in both ascites producing groups and the number of mice from which it was possible to collect ascites in two taps. There were included 889 mice in the study, 29.13% of them received the simultaneous injection of mineral oil and hybridoma cells after being primed with mineral oil. In this group, 97.68% of the mice developed ascitic tumors and it was possible to collect ascites from them, while from the group that received a single dose of mineral oil the ascites was collected in a smaller number of mice (90.47%). Ascites was not collected in 7.42% of all mice; the main cause was they never developed ascites tumors but no solid tumors were observed either. Within this percentage, the minority fraction, 9.09%, corresponded to the group 2. The observations performed by personnel familiar with the clinical signs associated with the production of ascites, did not report differences in the behavior and appearance of the mice, related to the hybridoma cell lines that were inoculated, which means that none of these lines was particularly aggressive to the well-being of mice.

Table 1. Distribution of the mice inoculated with hybridoma cell lines and the mice that produced ascites in both groups under study.

Hybridoma cell lines	No. mice injected		No. mice collected	
	Group 1	Group 2	Group 1	Group 2
CBSSNCOV.2	130	10	124	10
CBSSNCOV.3	100	130	93	126
CBSSNCOV.10	120	50	117	50
CBSSRBD-S.1	150	25	120	23
CBSSRBD-S.2	50	24	39	24
CBSSRBD-S.3	80	20	77	20

Group 1: Mice were inoculated intraperitoneally with $2x10^6$ cells of each hybridoma suspended in 1 mL of RPMI-1640 medium, 7 days after priming with 0.5 mL of mineral oil.
Group 2: Mice were inoculated simultaneously with the same inoculum of hybridoma cells and 0.5 mL of mineral oil, 18 days after priming with 0.5 mL of mineral oil.

The volume of ascites per mouse was calculated by dividing the total volume of ascites harvested by the number of mice collected in each group. The values ranged from 1.38 to 4.90 mL per animal. The independent samples t-test demonstrated that both groups under study were significant different in terms of the volume of as-

cites per mouse. Simultaneous injection of mice with mineral oil and hybridoma cells increased these volumes in all cases with respect to the mice injected with a single dose of mineral oil (Figure 1). The mean ascites volume per mouse in this group was 1.75-fold higher than the estimated mean for group 1.

Fluctuations in the IgG concentration quantified in clarified ascites, both within and between groups, were observed (Table 2). Nevertheless, the analysis of the data from both groups following an independent samples t-test, showed no significant differences in the mean IgG concentration in clarified ascites between both groups (t=1.115; p=0.291). In addition, an approximation of the MAb mass yield per mouse was also similar, 17.64 mg for group 1 and 16.48 mg for group 2.

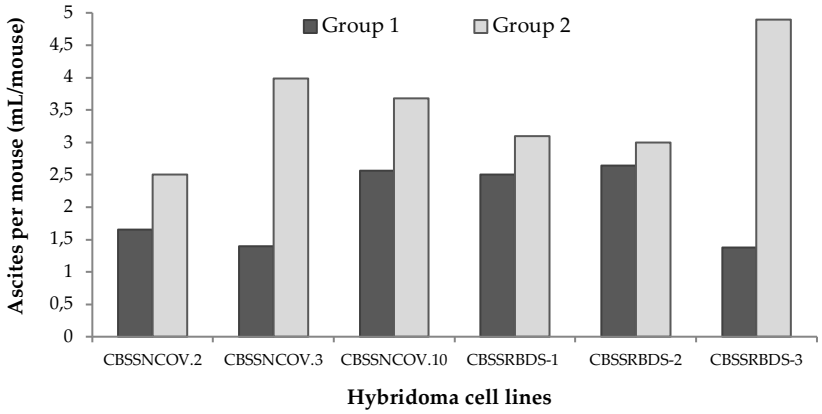

Figure 1. Volume of ascites produced per mouse in both groups under study. All mice were primed with 0.5 mL of mineral oil into the abdominal cavity. Group 1: Seven days after priming, mice were inoculated intraperitoneally with $2x10^6$ cells of each hybridoma suspended in 1 mL of RPMI-1640 medium. Group 2: Eighteen days after priming, mice were inoculated simultaneously with the same inoculum of hybridoma cells and 0.5 mL of mineral oil. The independent samples t-test showed that the volume of ascites per mouse was significant different between the groups (t= -3.747; p= 0.004).

Table 2. Volume of clarified ascites and resultant quantity of IgG from each hybridoma cell line inoculated in both groups of mice.

Hybridoma cell lines	Clarified ascites volume (mL)		IgG in ascites (mg/mL)	
	Group 1	Group 2	Group 1	Group 2
CBSSNCOV.2	215	25	9.06	8.56
CBSSNCOV.3	187	280	7.19	9.05
CBSSNCOV.10	300	82	9.95	6.28
CBSSRBD-S.1	300	72	2.49	5.81
CBSSRBD-S.2	103	72	13.04	3.64
CBSSRBD-S.3	265	98	6.83	3.67

Group 1: Mice were inoculated intraperitoneally with $2x10^6$ cells of each hybridoma suspended in 1 mL of RPMI-1640 medium, 7 days after priming with 0.5 mL of mineral oil.
Group 2: Mice were inoculated simultaneously with the same inoculum of hybridoma cells and 0.5 mL of mineral oil, 18 days after priming with 0.5 mL of mineral oil.

4. Discussion

In this work we assayed two strategies for production of murine ascites containing anti-SARS-CoV-2 MAbs using BALB/c mice. Since sensitive techniques have not been developed to measure signs that might indicate the presence of pain or distress [6], one of the most important tasks in the present study was the daily observation of the mice involved, including the examination and palpation of the injection site, the evaluation of abdominal distention and the detection of all possible side effects of the injected mixture. Some hybridoma cell lines can produce clinical signs in mice indicating distress, such as anorexia, hunched posture, hypothermia, rapid breathing and decreased activity [6]. But none of the six hybridoma cell lines used in this work caused worrisome adverse effects as a sign of an aggressive cell line, and this was an important ethical advantage.

Regarding the timing of priming agent administration in relation to the inoculation of the hybridoma cells, it is common practice to perform priming and several days later to inject the hybridoma cell-suspension into the peritoneal cavity of the mouse. This leads to the development of a tumor, the accumulation of ascitic fluid and the abdominal distention [2, 6]. However, it is not usual the simultaneous administration of the hybridoma cells with an additional dose of the priming agent. In the present study, the objective of the second administration of the mineral oil was to increase the throughput in the production of ascites. Mineral oil is thought to act by inducing granulomatous inflammation and interfering with peritoneal lymphatic drainage, thus increasing the volume of ascites produced [7]. We consider that the purpose was achieved because it was possible almost to double the volume of ascites per mouse in the group that received the second injection of mineral oil. This procedure could cause animal distress, but it did not occur in the mice included in this work. Some authors have evaluated the effects of priming agent injection on mouse well-being and ascites production, using parameters related with the mouse activity and food and water consumption [8]. In our study, the observation of mice at least twice a day by qualified personnel, did not inform significant evidences of distress or pain in the animals, since they maintained their normal activities and external appearance until the second tap. The volume of ascitic fluid did not cause gross abdominal distention even when a double volume of the priming agent was administered; moreover, the abdominal tap was done before fluid accumulation became excessive and distressful, following the recommendations that it not exceed 20% of the baseline body weight of the mouse [6].

Ascites yields can be improved also by performing several harvests; nevertheless, the well-being of mice must be strictly observed. The number of taps should be restricted according to animal welfare and characteristics of the hybridoma being used. Some hybridomas seem to cause little distress and various taps could be permissible [9], but the prolongation of tapping time increases the pathological abnormalities in the mice due to solid tumor growth within the peritoneal cavity and the accumulation of ascites [10]. Various guidelines and reports have required that the abdomen may be tapped no more than twice before the mouse is euthanized for final harvest of ascites [2, 8, 11]. The present study was careful with this aspect, since ascites pressure was relieved before abdominal distention was great enough to cause discomfort or disturb the normal activity of the animals, and only two taps were performed to obtain ascites, despite the fact that the hybridomas inoculated did not cause adverse effects in mice.

A crucial finding was that there were no significant differences in the concentration of IgG in ascites and in the mass yield of MAb per mouse between the groups analyzed. According to these results, in order to obtain a greater mass of MAb, the strategy applied in the group of mice that significantly increased the volume of ascites fluid per mouse must be taken into account: the simultaneous injection of hybridoma cells and mineral oil eighteen days after priming. Furthermore, the lowest percentage of mice that never developed ascites tumors was found in this group. Consequently, this strategy could allow the usage of fewer animals for the production of ascites. It is clear that the number of mice to be used will be determined by the total amount of MAbs needed [10]; but, certainly, the reduction of the amount of animals required for the MAbs production offers important ethical and economic advantages.

Medium-scale production of MAbs is demanded to make 0.1-1.0 g quantities for diagnostic or developing assays [3]. Applying the strategy used in group 2 it was possible to produce more quantities than 1.0 g of each MAb. For example, mice included in this group produced approximately 4.0 g of CBSSNCOV.3 and more than 1.0 g of CBSSNCOV.10. Both MAbs are being used in the ELISA developed in our country for the diagnosis of SARS-CoV-2 by detecting viral antigen, and they are constantly demanded for immediate diagnosis as urgent necessity of current epidemiological scenario. Therefore, with the simultaneous injection of hybridoma cells and mineral oil in previously primed mice, it is possible to guarantee the production of required quantities of both MAbs with a minimum number of animals involved.

The MAbs against the RBD of the SARS-CoV-2 spike protein (CBSSRBD-S.1, CBSSRBD-S.2 and CBSSRBD-S.3) could play an important role in subunit vaccines development. For this reason, it is necessary to optimize all the productive stages to ensure the availability of these MAbs for future assays, and this work also contributes to this.

5. Conclusions

The double administration of mineral oil, for priming and for the inoculation of hybridoma cells, does not produce additional stress in the mice. None of the six anti-SARS-CoV-2 MAb-secreting hybridoma cell lines, which were injected to mice, are harmful to the animal well-being. The simultaneous inoculation of the hybridoma cells with the mineral oil to the primed mice improves the efficiency of ascites tumor formation and significantly increases the volume of ascites per mouse. This inoculation strategy will optimize the production of ascites with anti-SARS-CoV-2 MAbs, involving fewer animals, which translates into ethical and economic benefits.

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Conflicts of Interest: The authors declare no conflict of interest.

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Communication

Glaucoma in dogs

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Abstract: Glaucoma is a group of diseases commonly defined by an increased intraocular pressure (IOP) which interferes with normal function of the optic nerve and retina. Characteristic changes of glaucoma include reduced axoplasmic flow in the optic nerve head, retinal ganglion cells death, cupping of the optic disc and visual damage or blindness due to retinal and optic nerve atrophy [1].

Keywords: glaucoma, menace response, gonioscopy, dogs

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1. Introduction

According to the European College of Veterinary Ophthalmologists (www.ecvo.org) Manual 'Glaucoma is characterized by an elevation of intraocular pressure (IOP) which, when sustained, results in destruction of the intraocular structures and function, resulting in blindness. The elevated intraocular pressure occurs mainly with developmental abnormalities or disease processes affecting the intraocular circulation and especially the drainage of aqueous humour from the eye through the irido-corneal angle (ICA). Open Angle Glaucoma (POAG) in specific breeds are available.'

Glaucoma typically occurs as a result of impaired aqueous humor drainage whereby the defect in the drainage pathway may lie at the level of either the pupil (pupil block glaucoma), the ICA (trabecular meshwork, the conventional pathway) or uveoscleral outflow (unconventional pathway) [2].

The main function of the aqueous humor is to provide nutrients to the cornea and lens. The aqueous humor is constantly produced at the level of the non-pigmented epithelium of the ciliary processes (Fig. 1). It flows through the posterior chamber, the pupil and into the anterior chamber (dorsal to ventral direction due to thermal convection currents) to be drained into the ICA (the filtration angle, the anterior most portion of the ciliary body, Fig 2.). The ICA is formed by the junction of the inner cornea (corneoscleral tunic), base of the iris and ciliioscleral cleft (which contains the pectinate ligaments). The aqueous humor is then drained through the trabecular meshwork and associated aqueous collecting channels (the conventional and main pathway) and less through the ciliary body and anterior uvea (the uveoscleral pathway, also called the unconventional/alternative pathway).

The entire anterior chamber volume (0.4 ml in the dog) is replaced within an hour in most species (46-80 minutes in the dog). Therefore, the IOP is the result of the balance

between production and outflow of aqueous humor. The production of aqueous humor is a dynamic process, the inflow of aqueous humor equals the outflow.

In glaucoma, both the aqueous humor production and outflow are altered. In understanding the pathogenesis of glaucoma, it is very important that the anatomy and physiology of the aqueous humor outflow pathways are well understood in normal and glaucomatous eyes.

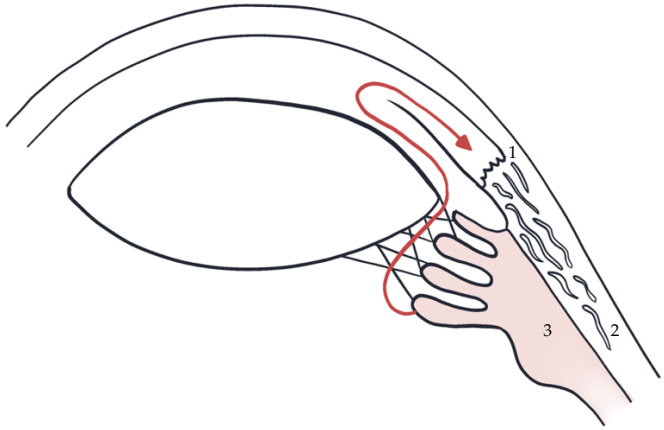


Figure 1. Aqueous humor drainage routes in canine eye. After being produced at the level of the ciliary processes, the aqueous humor flows through the posterior chamber into the anterior chamber (dorso-ventral direction, via convection currents). Here, it is drained via the trabecular meshwork of the ciliary cleft and into the angular aqueous plexus then directed anteriorly into the episcleral venules (1) or posteriorly into the scleral venous plexus and the vortex venous system (2). An alternative aqueous humor drainage pathway (3) is the diffusion through the ciliary muscle interstitium to the suprachoroidal space and through the sclera (ie, uveoscleral outflow).

Source: Original AZ adapted after [3]

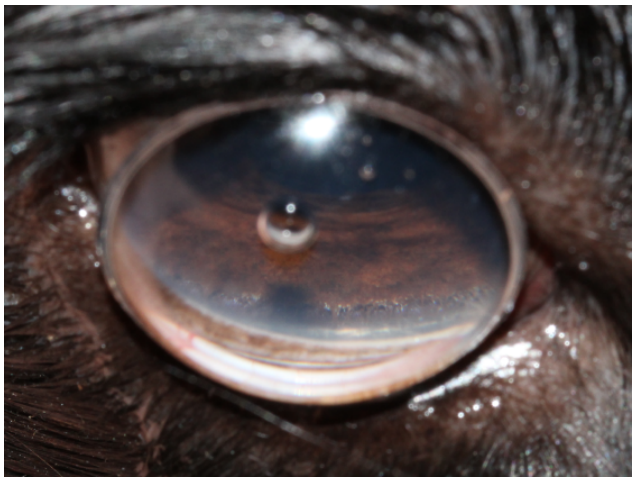


Figure 2. The iridocorneal angle visualized by gonioscopy. 8 year old cocker spaniel with primary closed angle glaucoma. (Royal Veterinary College Archive)

2. Causes and clinical signs of glaucoma

Glaucomas have been classified according to their cause into congenital, primary or breed-related (open angle and closed angle glaucoma) and secondary to other intra-ocular diseases (eg. chronic uveitis, primary lens

luxation and intraocular neoplasia, Table 1). The canine glaucomas can also be classified according to the stage of progression into acute or chronic.

Table 1. Causes of glaucoma according to [2]

Congenital glaucoma	Goniodisgenesis associated with multiple ocular defects
Primary glaucoma	Goniodysgenesis/narrow/closed irido-corneal angle: Breeds affected www.ecvo.org: • Japanese Shiba Inu • Dandie Dinmont terrier • Leonberger • Retriever (Flat Coated) • Siberian Husky • Spaniel (American Cocker) • Spaniel (Cocker) • Spaniel (English Springer) • Spaniel (Welsh Springer) • Spanish Water Dog
	Primary open angle glaucoma - Beagle, Norwegian Elkhound, Petit Basset Griffon Vendéen, Basset Hound, Shar Pei
Secondary glaucoma	Formation of pre-iridal fibrovascular membranes (PIFM) over the ciliary cleft opening - secondary to retinal detachment, neoplasia or uveitis
	Intraocular haemorrhage (due to systemic hypertension, coagulopathies, thrombocytopaenia)
	Intumescence of a cataractous lens (phacomorphic glaucoma in diabetic cataracts)
	Uveal cysts and iridiociliary glaucoma in the Golden Retrievers
	Intraocular neoplasia
	Postoperative following cataract surgery
	Ocular melanosis - pigmentary glaucoma in Cairn Terriers
	Primary lens luxation - terrier breeds, Border collie etc.
	Uveitis, including lens - induced uveitis
	Vitreous prolapse after surgical lens extraction (due to obstruction of the drainage angle by the vitreous)

Clinical signs of glaucoma vary with the duration, intensity and cause of the IOP elevation from simple ocular surface redness (conjunctival hyperaemia and episcleral congestion that can be confused with conjunctivitis

or retrobulbar disease), third eyelid protrusion and ocular discharge (commonly mistaken for conjunctivitis) to diffuse corneal oedema (cloudiness) that comes and goes (due to intermittent pressure spikes) and enlarged globe. Visual impairment may not be obvious to the owner as the dog will likely rely on vision from the contralateral eye, however the owner may report that the dog had been bumping into things on a particular side, missing things or being startled when approached from a particular side.

As the dog is presented with a red eye, other ocular diseases should be included on the differential list such as uveitis (Fig. 3), episcleritis, conjunctivitis, retrobulbar disease. It is not uncommon to misdiagnose glaucoma as conjunctivitis which leads to irreversible blindness due to the delay in the appropriate treatment. Distinguishing between various diseases that can cause redness should be based on the result of the clinical examination (the presence of menace response, pupillary light and dazzle reflexes) and tonometry (if available).

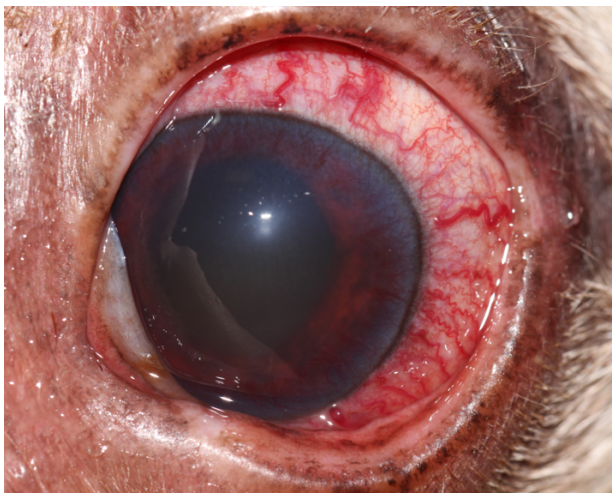


Figure 3. Uveitis and secondary glaucoma, Labrador, 7yo, MN- note conjunctival hyperamia, episcleral congestion, perilimbal deep corneal vascularisation, subtle perilimbal corneal oedema, mid-dilated pupil, iris vasodilation (Royal Veterinary College Archive)

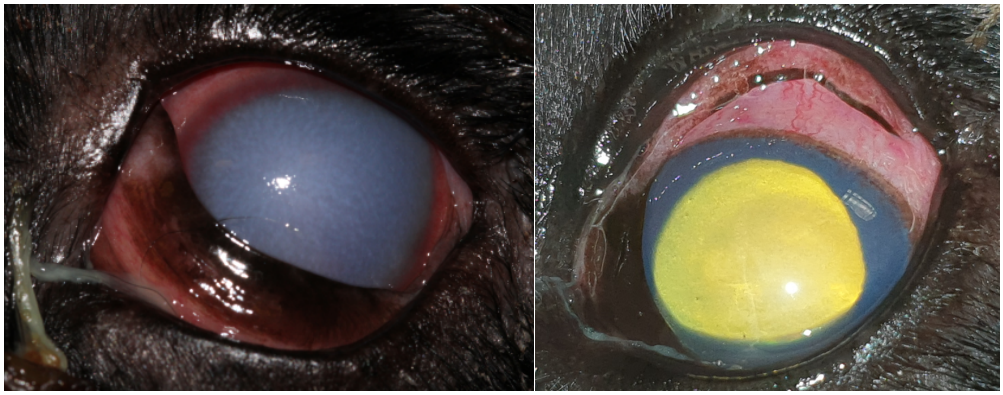
Primary sudden onset congestive glaucoma is easier to recognize as the dog will be typically middle age, female and will have absent menace response, fixed dilated pupil +/- absent dazzle reflexes, diffuse corneal oedema, episcleral congestion and conjunctival hyperaemia (Fig. 4). Cases of secondary glaucoma are more difficult to recognize solely based on the clinical signs but one should carefully look for signs of uveitis, retinal detachment or intraocular neoplasia, mid-dilated pupil with absent PLR and reduced or absent menace response.

Figure 4. English Springer Spaniel, female- Primary glaucoma left eye, note dilated pupil, diffuse corneal oedema, perilimbal corneal vascularisation, conjunctival hyperaemia, episcleral congestion (Royal Veterinary College Archive)

Clinical signs of acute onset glaucoma (more common in primary glaucoma cases)

- Severe ocular and head pain;
- Lethargy, reduced appetite;
- Diffuse corneal oedema (Fig. 5);

- Blepharospasm;
- Epiphora;
- Marked conjunctival hyperaemia which can be misdiagnosed as conjunctivitis;
- Third eyelid protrusion;
- Marked episcleral vascular congestion;
- Mid to large dilated and unresponsive pupil;
- Deep perilimbal corneal vascularisation;
- Mild aqueous flare or pigment dispersion in the anterior chamber
- Changes of the optic nerve appearance: pale or dark optic nerve head (atrophy), attenuation of the retinal vasculature, cupped optic nerve head (retinal vessels stop at the rim of the optic nerve, no vessels are seen crossing the optic nerve), hyperreflectivity of the tapetal fundus.



(a) (**b**)

Figure 5. 8yo Cocker spaniel (both images), female neutered with primary angle closed glaucoma- mucoid discharge at the medial canthus, third eyelid protrusion, perilimbal deep corneal vascularisation, conjunctival hyperaemia, diffuse corneal edema (North Downs Specialist Referrals)

Clinical signs of chronic glaucoma (more common in secondary glaucoma):

- Development of Haab' striae (Fig. 6): breaks in the Descemet membranes (allowing aqueous humor to enter the posterior stroma) secondary to stretching of the globe.
- Buphthalmos: physical enlargement of the globe, resulting from severe and chronic elevations in IOP. It may be especially pronounced in young animals and Shar Pei dogs (with POAG), who have a more easily distended cornea and sclera than most adult dogs; buphthalmic eyes are almost invariably blind [1].
- Scleral thinning and visualization of the underneath choroid due to globe enlargement.
- Phthisis bulbi: in advanced cases when the ciliary body stops producing aqueous humor.

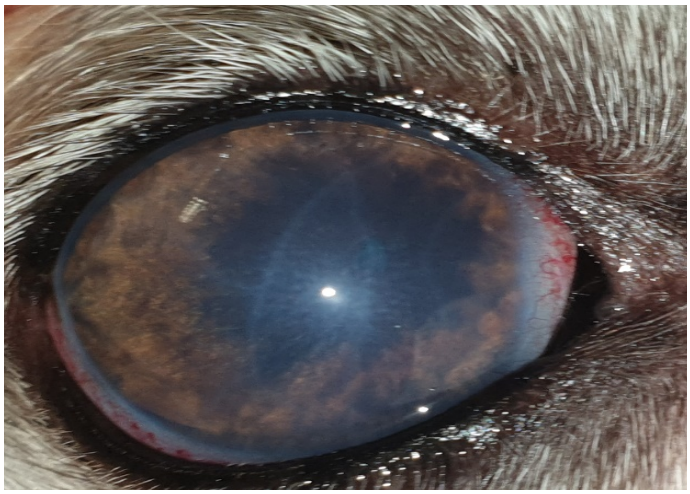


Figure 6. 6yo FN Whippet- Anterior uveitis and secondary chronic glaucoma- note the Haab's striae (curvilinear grey/white lines across the cornea), the pupil was miotic owing to the recent application of latanoprost (North Downs Specialist Referrals)

Congenital glaucoma

Congenital glaucoma is a rare condition which develops due to severe ocular malformation and extensive goniodysgenesis associated with multiple ocular defects. Affected animals display severe, bilateral, ocular pathology, very early in life (generally within weeks to months) [3].

Primary glaucoma

Primary glaucoma is a bilateral disease which is not associated with any pre-existing intraocular disease, but has been associated with dysgenesis of the mesenchymal structures of the ICA (pectinate ligament dysplasia/goniodysgenesis) and/or narrowing or closure of the iridocorneal angle and/or the ciliary cleft (CC) in some breeds [6].

Clinical signs usually do not become evident until relatively late in life and this could be due to age-related changes in the ICA. Primary glaucoma is a bilateral disease, however the onset of disease varies between the eyes [6].

Based on the appearance of the drainage angle (gonioscopy), primary glaucoma may be classified as open-angle glaucoma (Beagle, Petit Basset Griffon Vendeen, Basset Hound, Shar Pei, Norwegian Elkhound) and closed angle glaucoma more common in the majority of breeds (Cocker spaniel, Welsh springer spaniel etc). When the ICA appears abnormal on gonioscopy then goniodysgenesis has been diagnosed. A defect in the development of the ICA leads to a decreased width or malformation of the pectinate ligament. It is advised against breeding of dogs diagnosed with PCAG.

Typical presentation of a dog with PCAG is a sudden onset of unilateral acute congestive glaucoma (sudden blindness, dilated pupil, third eyelid protrusion, diffuse corneal oedema, marked episcleral congestion) in a middle aged dog (6-8 years of age) more common in female dogs belonging to one of the following breeds: American/English Cocker spaniel, Springer spaniel, Beagle, Boston Terrier, Norwegian elkhound (full list of affected breeds available at https://www.bva.co.uk/canine-health-schemes/eye-scheme/ or https://www.ecvo.org/hereditary-eye-diseases/ecvo-manual.html to check if a specific breed can be affected by primary glaucoma). The second eye typically follows the same outcome within 6-12 months after diagnosis.

Bilateral presentation of PCAG is rare and other intraocular conditions should be considered particularly uveitis/panuveitis, uveo-dermatologic syndrome, chorioretinitis and retinal detachment when a dog is presented with bilateral high intraocular pressures.

Secondary glaucoma (post-inflammatory)

Describes elevated intraocular pressure that occurs secondary to underlying ocular disease. Common causes of secondary glaucoma include cataract (Fig. 7), post-cataract surgery, lens (sub)luxation, intraocular neoplasia, severe or chronic uveitis, retinal detachment, lens-induced uveitis, uveo-dermatologic syndrome.

Secondary glaucoma can develop acutely, subacutely, or chronically and may affect one or both eyes. Clinical signs include ocular discomfort, blepharospasm, corneal oedema, episcleral congestion (Fig 8), third eyelid protrusion, miosis or mydriasis, uveitis, buphthalmos and/or blindness [4,5]. Cases of glaucoma may be mistaken for conjunctivitis or retrobulbar disease due to the conjunctival hyperaemia, third eyelid protrusion and ocular discharge.

Figure 7. 13yo FN Miniature Schnauzer- chronic lens-induced uveitis and secondary pupil block glaucoma, under treatment- pigment dispersion on the surface of the iris and anterior lens capsule, posterior synechiae, fixed pupil (North Downs Specialist Referrals)

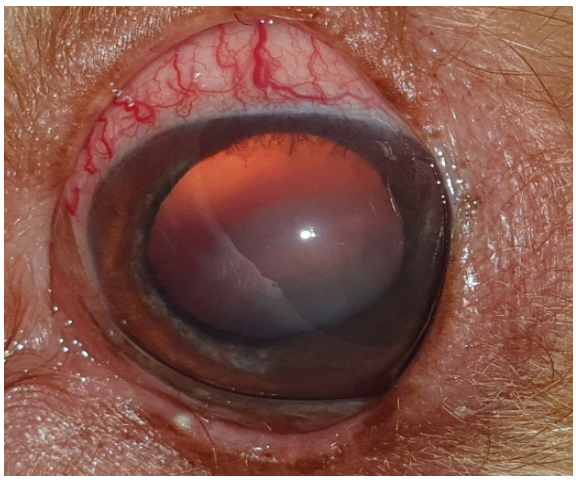


Figure 8. 8yo FN Miniature Poodle - posterior lens luxation and secondary glaucoma. Note the conjunctival hyperaemia and engorged episcleral vessels. (North Downs Specialist Referrals)

Pigmentary glaucoma

Pigmentary glaucoma (ocular melanosis) is a form of glaucoma which occurs as a result of the proliferation and accumulation of cells containing melanin in the aqueous outflow. Studies support a hereditary aetiology. Ocular signs are generally bilateral, although not always symmetrical. Commonly affected breeds include the Cairn Terrier, Boxer and Labrador Retriever.

3. Diagnosis of glaucoma in the dog

Vision loss can happen gradually over a few weeks or months or acutely in dogs with glaucoma. Ophthalmic examination, including funduscopy, should be performed to rule out other ocular causes such as retinal disease or uveitis. The history, clinical presentation and full ophthalmic examination findings support the diagnosis of glaucoma. IOP values greater than 25 mmHg are suspicious for this condition.

For the evaluation of visual function the most important part is the assessment of the PLRs *(*pupillary light reflexes*).* It is important to evaluate the size of both pupils, and note any difference in pupil size (anisocoria). With raised intraocular pressure the PLR function is affected, typically the pupil is fixed and dilated.

Evaluating the menace response (Fig. 9) is another important step of the ophthalmic examination. This response is elicited by a threatening hand gesture heading towards the eye. A blinking response and globe retraction are expected to occur. This response involves cerebral cortical integration and interpretation, therefore it is not a reflex. It requires the entire peripheral (retina, optic nerve) and central visual pathways, as well as the visual cortex and the facial nucleus of cranial nerve VII, to be intact [7,8].

Figure 9. Evaluation of the menace response (North Downs Specialist Referrals)

To avoid **false positive** response from the visual, contralateral eye, the menace response should be evaluated in one eye, while the other eye is covered. It is important not to touch the eyelashes/hair of the patient, or cause air movement, as these may also elicit false positive response. A facial nerve paralysis may cause a **false negative** response. Therefore, in the absence of a menace response it is better to test the blinking reflex by tapping the skin at the canthus. The menace response is absent in very young (<10–12 weeks) animals, and may also be affected by the mental state of the patient [1,8].

In glaucoma the menace response will be either reduced/inconsistent or absent, due to the retinal ganglion cells damage and reduced axoplasmic flow. If the high intraocular pressure persists the damage can be irreversible leading to blindness.

The *dazzle reflex* (Fig. 10) is a subcortical reflex (mediated by reflex centers in the midbrain with fibers to the facial nucleus) whereby a strong light shone into the eye leads to blinking, globe retraction, third eyelid protrusion, and/or head movement. This is helpful when the ocular media are opaque (hyphaema, cataract) and when

the menace response and/or PLRs can't be evaluated. However, it doesn't indicate that the eye is visual, as this reflex can still be present even after retinal detachment due to the presence of a subspecialized population of retinal ganglion cells that are involved in the control of the PLR. Though, when the dazzle reflex is absent in a dog with high IOP it most likely indicates the retinal damage and unless immediate action is taken (medical treatment/aqueous centesis), blindness is likely to be permanent.

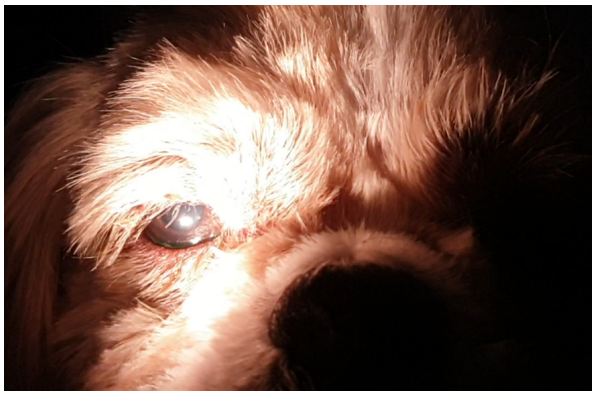


Figure 10. Assessment of dazzle and pupillary light reflexes (North Downs Specialist Referrals)

1. **Tonometry**: there are three methods of evaluating the intraocular pressure in animals: the indentation tonometer (Schiøtz) which is not routinely available in clinical practice, applanation tonometer (TonoPen) and the rebound tonometer (TonoVet, Fig. 11). Tonometry is also useful for identifying low IOP, which occurs with anterior uveitis; normal values vary between individuals and time of the day (diurnal variation). Normal reported range for the intraocular pressure in dogs is 10-20 mmHg.

Figure 11. Estimation of intraocular pressure using the TonoVet (rebound tonometer) North Downs Specialist Referrals

2. **Ophthalmoscopy**: direct and indirect ophthalmoscopy (Fig. 12) may be used to examine the retina (look for hyperreflective striations or generalized hyperreflectivity) and the health of the optic nerve (Fig. 13, normally the optic nerve head should be well vascularised, the blood vessels should be seen crossing its border). Optic disc cupping (the blood vessels stop at the rim of the optic disc due to increased pressure) is the hallmark of glaucoma[1]; however, this may be difficult to notice in cases of acute congestive glaucoma due to the marked corneal oedema.

Figure 12. Indirect ophthalmoscopy using a 30 D Volk lens- note the optic disc at the top with the retinal vessels emerging from it, tapetal fundus at the bottom (the image is inverted in indirect ophthalmoscopy) North Downs Specialist Referrals

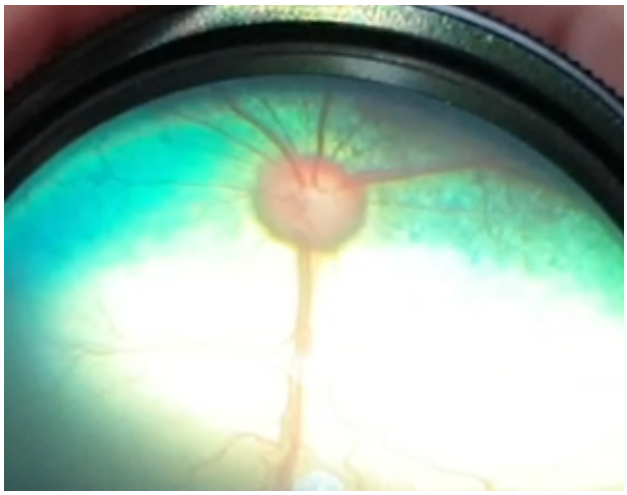


Figure 13. Normal appearance of the optic disc - pink with a physiologic pit centrally, retinal vessels crossing the border (North Downs Specialist Referrals)

3. **Gonioscopy:** visualization of the pectinate ligament using a goniolens (eg. Koeppe lens) which refracts the incoming light in such a way that allows visualization of the posterior cornea, ICA and anterior iris. This test is performed on the conscious dog under local anaesthetic and is used to diagnose goniodysgenesis (predisposition factor for primary glaucoma in a few breeds of dogs); it also provides information regarding the anatomy of the pectinate ligament which makes up the ICA.

There are three categories of ICA appearance (ECVO scheme): open, narrow or closed angle; with this test you can also visualise the base of the iris, pectinate ligaments and the base of the cornea. Abnormalities of the pectinate ligament may be classified into: fibre latae and laminae. Depending on the % of the affected area within the pectinate ligament the dog may be classed as unaffected or affected (mild, moderate or severe).

4. **Ocular ultrasonography**: used to rule out intraocular neoplasia, haemorrhage, vitriitis/vitreous membranes and retinal detachement.
5. **Ultrasound biomicroscopy**: high frequency (50-100 MHz) ultrasound, useful to visualize the width of the ciliary cleft/ the opening of the iridocorneal angle [11].

Other tests are available and are not discussed here: chromatic PLR testing, electroretinography (pattern ERG to assess retinal ganglion cell function), optical coherence tomography (OCT, used to measure retinal layer and optic nerve head thickness), scanning laser polarimetry (evaluates retinal nerve fibre layer, used for early detection of glaucoma patients).

4. Discussion

There are two ways of dealing with glaucoma: medical and surgical treatment either alone or in combination. Very early diagnosis and aggressive therapy are generally required to preserve vision and delay the onset of blindness due to retinal and optic nerve degeneration.

In primary glaucoma, despite normalization of the IOP following medical management, the glaucoma continues to progress, thereby supporting the evidence that changes occur at a molecular level and the condition can only be delayed but not cured. Despite aggressive medical or surgical treatment, the outcome is always the same which is blindness and loss of the eye and this needs to be explained to the owner.

Unfortunately, by the time the owner notices changes in the eye, often the IOP exceeds 40 mmHg so glaucoma is a leading cause of irreversible vision loss in dogs. The patient is lethargic and reluctant to exercise and the affected eye becomes blind and appears red, painful and sore with a bluish tinge (corneal oedema) over the cornea. Often the eyes are enucleated because of the painfully high and uncontrolled IOP. Glaucomas can easily mask underlying systemic diseases, such as infectious uveitis and neoplasia (lymphoma). Submission of the enucleated eyes for histopathological examination is always recommended [9].

Because newly developed glaucoma medications are emerging at a very slow rate and may not be effective, working toward improving surgical options may be the most rewarding approach in the near term [10].

Treatment of primary glaucoma (table 2): acute glaucoma should be considered an emergency and the pressure must be reduced to the normal range in order to save the patient's vision; hospitalization is required and treatment is generally commenced with topical prostaglandin analogues. If the IOP doesn't returns to normal within the first 30–60 min then the use of osmotic diuretics or aqueous centesis should be considered.

In current clinical practice, ophthalmologists rarely use osmotic diuretics due to the availability of prostaglandin analogues which are as or even more efficient without the risk of inducing side effects. Furthermore, osmotic diuretics should be cautiously used as they are contraindicated in patients with kidney disease, owing to the risk of causing dehydration and electrolytes imbalance. The use of osmotic diuretics has been generally replaced by prostaglandin analogues (eg latanoprost) combined with topical carbonic anhydrase inhibitors (brinzolamide, dorzolamide) and beta-blockers (eg timolol).

Treatment of glaucoma should be aimed at two directions:

- Reducing the aqueous humor production: carbonic anhydrase inhibitors, beta-blockers
- Increasing the aqueous humor outflow: eg. prostaglandin analogues (eg. Latanoprost). One effect of this class of hypotensive is the secondary miosis as well as conjunctival hyperaemia, due to the increased vasodilation (Fig. 14).

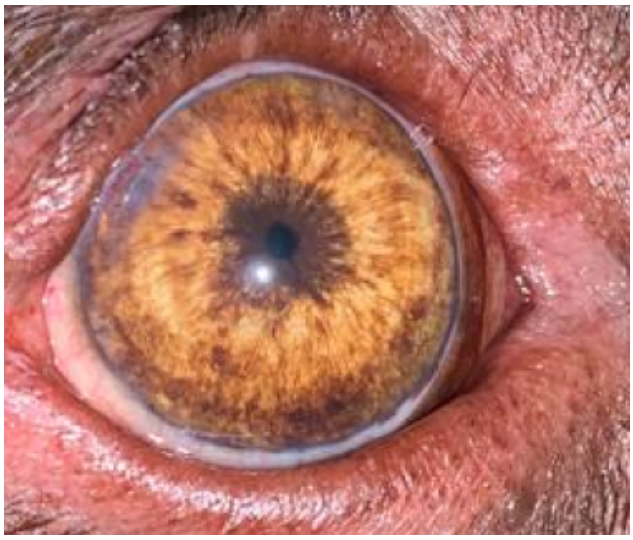


Figure 14. The effect of latanoprost application, leading to a very miotic pupil (North Downs Specialist Referrals)

Although there is no clear-cut evidence, long-term anti-glaucoma medication (e.g. with topical carbonic anhydrase inhibitors q8 hours) for the second, predisposed, but normotensive eye should be considered [2].

Analgesia: an IOP over 35 mmHg will cause migraines in people. Dogs with glaucoma are typically less active, lethargic, they sleep more, eat less and are less willing to go for walks. Analgesia should be provided in the form of systemic non-steroidal medication, paracetamol and/or opioids if there are no contraindications.

Treatment of secondary glaucoma depends on the etiology; therapy may consist in removal of the lens in cases of primary lens luxation to enucleation for those secondary to an intraocular tumor, but in all cases referral to a veterinary ophthalmologist should always be considered and discussed with the owner.

Table 2. Most commonly used hypotensive drugs for glaucoma in dogs and cats
Source: Adapted after [12]

Drug	Available Preparations	Dose or Timing	Contraindication
Osmotic diuretics			
Mannitol	20% solution Only in acute congestive glaucoma	1-1.5 g/kg i.v. slowly over 20 min	cardiac or renal disease, dehydration, chronic glaucoma
Prostaglandin analogues			
Latanoprost	0.005% solution	q12-24 h can be increased to q6-8 hours until a response is seen	severe uveitis, anterior lens luxation
Travoprost	0.004% solution		
Bimatoprost	0.03% solution		
Carbonic anhydrase inhibitors			
Brinzolamide	1% solution	q8-12h	none in dogs, but may cause local irritation shortly after instillation; keratitis, corneal oedema, blepharitis systemic absorption can lead to acute kidney injury in cats (check electrolytes before starting treatment) [13,14]
Dorzolamide	2% solution		
β -adrenergic blockers			
Timolol maleate	0.25% and 0.5% solution	Q8-12h	cardiac or respiratory disease use cautiously in cats and small dogs due to systemic absorption, cautious in dogs with asthma and chronic bronchitis

5. Conclusions

Despite medical therapy, a significant proportion of cases may require surgery in an attempt to keep the IOP within the normal range. The prognosis depends to the underlying cause of glaucoma. Long-term therapy is necessary and regular intraocular pressure checks are required in order to ensure a normal IOP. Eyes that have lost vision but still have an increased pressure are a source of chronic pain. In such cases, removal of the eye must be considered to guarantee the well-being and comfort of the patient. Due to the aggressive and progressive nature of the disease, some animals lose vision despite treatment. There are still options for blind and painful eyes, including eye removal with or without placing an intraocular prosthesis.

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CVJ CLUJ VETERINARY JOURNAL

Review

The link between mammary cancer, excessive adipose tissue and cholesterol

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Abstract: Mammary cancer remains the most frequent worldwide type of cancer in females. From a health point of view, it is a huge challenge. As a definition, we can say that a group of biologically and molecularly heterogeneous diseases is represented by mammary cancer. An important causal factor for this disease is genetic predisposition, especially mutations in the BRCA1 or BRCA2 gene. The mammary gland is stimulated by hormones both morphologically and physiologically. The most significant of these are estrogens.

Estrogen is the main female hormone, but it is present in both females and males. Elevated levels of this hormone may increase the risk of developing mammary cancer. In post-climacteric excessive adipose tissue, estrogens biosynthesis is catalyzed by aromatase, converting adrenal androgens into estrogen. Risk factors for developing mammary cancer, such as excessive adipose tissue, age at menarche and the use of exogenous hormones may increase the risk of developing it.

The aim of this paper is to show the link between cholesterol, excessive adipose tissue and the increased risk of developing mammary cancer.

Keywords: cancer, estrogen, hormones, cholesterol, obesity

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Introduction

Obesity is a risk factor for both mammary cancer as well as a strong prognosis, which predicts side effects of the disease. Clinical evidence shows that obese patients with mammary cancer who are being treated with chemotherapy or aromatase inhibitors are more likely to have a recurrence of the disease compare to females with normal body mass index [1]. Information about adipose tissue has increased significantly in recent years. Although adipose tissue has always been described for lipid storage, it is now identified as a true organ that possesses both metabolic and endocrine functions. It releases a variety of substances into the bloodstream to communicate with other organs and tissues [2].

Adipokines, adipose tissue-specific substances, are essential in determining a group of physiological responses, namely glucose and lipid metabolism, homeostasis, angiogenesis, inflammation and satiety [3]. On one hand, disorder of the hormonal role and uncontrolled expression of adipokines in adipose tissue, causes overweight or obese, eventually connecting obesity with mammary cancer risk [4]. On the other hand, there are studies that show that increased adiposity induces the growth and advancement of mammary cancer in post-climacteric females through the secretion of estrogen [4].

2. The role and effects of estrogens

The main roles of estrogen are to induce cell proliferation and the development of genitals and other tissues involved in reproduction [5]. Estrogens help with:

1. Development of the stromal tissue of the breasts;
2. Formation of a well-represented pipeline system;
3. Deposition of adipose tissue in the breasts.

The mammary lobes and alveoli develop only to a small extent under the influence of estrogen. Progesterone and prolactin are the hormones that have a decisive effect on the growth and functioning of these structures [6].

2.1 Effects of estrogen on metabolism and adipose tissue deposition - estrogen synthesis in obese adipose tissue.

Estrogen biosynthesis occurs primarily in the adipose tissue in post-climacteric females, through the conversion of adrenal androgens to estrogen by aromatase [7]. Several cellular and molecular changes in obese adipose tissue alter the biosynthesis and metabolism of estrogen.

Activation of the NF-κB pathway leads to an increase in aromatase expression in breast adipocytes and, therefore, to a higher estrogen synthesis (Figure 1) (Simpson et al., 2013). Similarly, several cytokines that are regulated in obese adipose tissue, such as TNF-α and IL-6, stimulate aromatase activity [8]. Excess breast fat, microscopic outbreaks of adipocytes surrounded by macrophages, present increased aromatase activity [9].

Moreover, in post-climacteric females, BMI is directly proportional to serial concentrations of estrone and estradiol and inversely proportional to hormone-binding globulin levels, leading to an increase in total bioavailable estrogen [7]. Compared to females with a BMI <22.5 kg / m2, obese females have an 86% increase in circulating estradiol, a 60% increase in estrone, and a 20% increase in testosterone [10].

3. Mammary cancer and excessive adipose tissue-mechanism

Chronic activation of NF-κB in adipose tissue not only causes obesity-mediated inflammation, but also stimulates anti-apoptotic genes and the proliferation of mammary cancer, invasion, angiogenesis and metastasis [11].

Concentrations of IL-6 in peritumoral fat are higher than in all other regions and grow with increasing tumor size and lymph node involvement. Some studies have shown that the interaction between cancer cells and adipocytes induces both cell types to increase the secretion of cytokines IL-6, IL-8, CCL2 and CCL5. In addition, this phenomenon promotes tumor invasion and metastasis [11, 12].

Adipose tissue normally helps stimulate up to 35% of circulating IL-6. It is also responsible for the increase in serum IL-6 after climacterium. In this way it can help increase the risk of mammary cancer and the progression of the tumor. IL-8 resulting from cancer cells, surrounding adipocytes, endothelial cells, infiltrating neutrophils, and tumor-associated macrophages (TAM) develops angiogenesis, tumor growth, metastasis, and resistance to chemotherapy [13].

4. The link between overweight and tumorigenesis

Along with obesity, the secreted cytokines go from an anti-inflammatory profile to a pro-inflammatory and proangiogenic profile. The secretion of pro-inflammatory and proangiogenic cytokines also increases in adipocytes, and ultimately results in the multiplication of cancer cells. In this way, the stimulation of angiogenesis, the expansion of cancer stem cells, invasion and metastasis takes place [14].

Excessive adipose tissue accumulates mediators of antitumor immunity, such as CD8-positive (CD8 +) T cells, natural killer (NK) cells, and dendritic cells, myeloid-derived suppressor cells (MDSC), and tumor-associated macrophages (TAM) which suppresses antitumor immunity (fig.1). In addition, stimulation of adipocyte aromatase results in higher estrogen synthesis and thus potentiates the development of estrogen receptor-positive (ER +) mammary cancer [13].

Adipocyte hypertrophy induces the secretion of inflammatory cytokines, chemokines, and leptin as a result of an increase in the number and size of adipocytes. These adipokines then induce macrophage recruitment and polarization. Macrophages secrete inflammatory cytokines, which can act directly to stimulate mammary cancer. In the end, all this leads to an increase in the production of aromatase and estrogen. In addition, they induce the expression of pro-angiogenic factors [13].

Inflammation of adipose tissue also promotes the development of insulin resistance, leading to the release of insulin and insulin-like growth factor (IGF). Insulin resistance and IGF can directly promote mammary cancer. Similarly, adipocyte leptin acts directly on cancer cells. Moreover, the decrease in adiponectin caused by excess adipose tissue has the same effect [13].

5. Cholesterol and the risk of mammary cancer

A number of measures have been implemented to detect and treat elevated cholesterol, largely through the use of statins (Figure 2) and more recently by PCSK-9 inhibitors [15]. PCSK9 inhibitors are monoclonal antibodies that inhibit proprotein convertase *subtilisin/kexin type 9* (PCSK9). PCSK9 is a protein that binds to LDL receptors for degradation and thus reduces the liver's ability to remove LDL-cholesterol from the blood, also called "bad" cholesterol [16].

The PCSK9 inhibitor is synthesized to bind to PCSK9 and prevent PCSK9 from binding to LDL (low density lipoprotein) receptors on the surface of liver cells. In the absence of PCSK9, there will be more LDL receptors on the surface of liver cells to remove LDL-cholesterol from the blood, resulting in a lower concentration of LDL cholesterol in the blood. In the last decade, there have been more and more results associating cholesterol with other modifiable risk factors, such as obesity and diabetes [17, 18]. Unfortunately, the combined action of these factors is reflected in a number of cancers, including mammary cancer [19].

High blood cholesterol is often associated with obesity [20]. Its impact as a risk factor in the occurrence of mammary cancer is not very clear and it has not been established which of the 3 parameters: total cholesterol, LDL or HDL contributes to the appearance of the disease [21]. The systematic analysis and meta-analysis of prospective studies that investigated the association between total cholesterol (CT), HDL-C, LDL-C, ApoA1 and ApoB and the risk of mammary cancer suggested an inversely proportional, statistically significant link [22].

At the end of the twentieth century, some epidemiological research (small sample size) studied the impact of serum cholesterol on the incidence of malignancy, but the results were inconclusive [23]. Others have suggested that red and processed meat, which contain a higher amount of LDL cholesterol, are risk factors for colorectal, mammary and endometrial cancer [24]. A larger study published in Science shows the role of cholesterol in the development of mammary cancer in mice. Moreover, the authors also showed that knockout mice in which cholesterol levels increased and were treated with statins had a lower predisposition to developing mammary cancer [25].

At the cellular level, it has been shown that there are many physiological mechanisms. Low serum cholesterol can increase the fluidity of the cell membrane which may result in the spread of cancer cells [26]. Conversely, the loss of membrane cholesterol can decrease the antigenicity of tumor cells, which results in the avoidance of the action of the immune system [27].

5.1 Cholesterol, HFD (high-fat diet), 27HC and cancer biology

Cholesterol represents a major factor mammary cancer risk. Unfortunately, the mechanism by which it takes place is not fully known. Most likely the increase in cholesterol content in cell membranes and after the affecting of the membrane fluidity caused by dyslipidemia may represent a possible explanation. Also, there are researches that show the functionality of the metabolite, 27-hydroxycholesterol (27HC) as an estrogen **[**21, 28]. The intensive proliferation of estrogen receptor (ER)-positive mammary cancer cells is a result of its action. In this way, the treatments used in mammary cancer in order to lower the concentration of cholesterol are justified [21].

In animals, the specific contribution of cholesterol, a comorbidity of obesity, in the pathogenesis of cancer was underestimated. This may be due to the fact that no increase in cholesterol was observed after a hyperlipidemic diet (HFD high-fat diet). To address this, a high-fat diet was used in humanized APOE3 mice. Circulating cholesterol levels were subsequently determined [25].

The observed increase in circulating cholesterol in HFD-fed mice is the result of intensified of novo synthesis. However, the effect of HFD on tumor growth was attenuated by statin treatment or inhibition of CYP27A1 [25]. These findings confirm the importance of cholesterol and dyslipidemia in mammary cancer and highlight the importance of 27HC and ER as mediators of these effects.

On the other hand, there are studies showing that the effect of statins in mammary cancer is not beneficial [29].

6. Conclusions

The effects of excessive adipose tissue on the risk of mammary cancer differ depending on the status of the ER. Obesity is associated with a significantly higher risk of ER-positive mammary cancer. The effect is insignificant for ER-negative mammary cancer after climacterium [30].

High concentrations of cytokines and leptins secreted by adipose tissue increase the number of preadipocytes, that release free fatty acids (FFA) and activate the NF-κB pathway. This pathway controls DNA transcription, cytokine production, and apoptosis in both adipocytes and immune cells in order to produce chronic inflammation [31]. The high number of preadipocytes causes high concentrations of IL-6, IL-8, CCL2, CCL5 and VEGF with a positive effect on the production of NF-κB and cytokines [32]. In addition, contact between adipocytes and invasive cancer cells synergistically regulates cytokine secretion. 9 TNF-α also called tumor necrosis factor alpha and IL-6 affects insulin receptor activation [33]. Finally, there is insulin resistance that has a positive impact on the development and growth of mammary cancer.

The NF-κB, TNF-α and IL-6 pathway also stimulates the expression of aromatase in stromal and adipocyte fibroblasts [8] breast. The effect is an increased estrogen production in both cancer and stromal cells. In addition to increasing insulin resistance, IGFs, adipokines, and local estrogen production, there is clear evidence to support the independent role of cholesterol as a mediator of the effects of dyslipidemia and/or obesity on the pathogenesis of mammary cancer [21].

A major mechanism by which cholesterol triggers mammary cancer includes its metabolite, 27HC, a molecule with SERM activity (which is a selective modulator of estrogen receptors). Studies highlight the immediate therapeutic potential for modulating cholesterol levels, either through diet or medication, such as statins, PCSK9 inhibitors, or niacin [34].

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, M.C. and P.B.; methodology,

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