Veterinary

Modern Analysis, Finding and Researches

Editors
Aliye SAĞKAN ÖZTÜRK
Atakan ÖZTÜRK

LIVRE DE LYON
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PREFACE

Veterinary medicine is a branch of medicine related to the prevention, management, diagnosis, and treatment of disease, and injury in animals. The synergy between human and veterinary medicine is not only a must for public health, but also implies preventive medicine, food safety. The relationship of the human and veterinary medicine is supported by the concepts of 'one medicine' and 'one health'. There are also many zoonotic diseases that spread between humans and animals. On the other hand, it is very important to conduct preliminary studies with experimental animals, especially in the evaluation of diagnosis and treatment in disease models. For this reason, veterinary medicine should both update itself and also follow technological and scientific developments.

The current book has been prepared with the aim of presenting the latest developments in veterinary medicine to readers.

I hope it will be useful.

Prof. Dr. Aliye SAĞKAN ÖZTÜRK
Asst. Prof. Dr. Atakan ÖZTÜRK
Editors

CONTENTS

CHAPTER I

INVESTIGATION OF THE EFFECTS OF METHYLPREDNISOLONE ON SERUM AMYLOID-A, TNF-A, IL-1B AND IL-6 RELATIONSHIPS IN THE AA AMYLOIDOSIS MODEL IN MICE

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1. Introduction

Amyloidosis is described as an extracellular and intracellular accumulation of characteristic fibril-formed amyloid proteins and has a typical X-ray diffraction pattern in electron microscopy and shows apple-green reflection under polarized light with Congo red stain affinity (1). In the formation of amyloidosis, occur the deposition of insoluble amyloid fibrils, which are aggregates formed from normally soluble proteins via conformational changes caused by various mechanisms (2,3). Secondary (reactive) amyloid A (AA) amyloidosis is a systemic type of amyloidosis associated with infectious, inflammatory, or, less commonly, neoplastic disorders and it is observed in domestic, laboratory, and wild animals, as well as in humans (4-9). Amyloidosis is identified with precursor proteins and several protein types (presently 36 in humans and 10 in other vertebrates) have been classified and more are to be expected (1). AA amyloidosis-related precursor protein is serum amyloid A (SAA), which is an acute-phase reactant mainly synthesized by hepatocytes (10). During the acute-phase response, there is a dramatic increase in SAA in plasma high-density

lipoproteins (HDL). Elevated SAA leads to reactive AA amyloidosis in animals and humans (11). Chronic infections or prolonged inflammatory conditions promote the release of pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-1β, and tumor necrosis factor-α (TNF-α) to trigger a thousandfold increase in the levels of SAA (8,12). After structural changes, the SAA cleavage product accumulates into highly organized protein fibrils with a β-pleated sheet known as amyloid (13-15). SAA deposits as amyloid fibrils and causes tissue structure damage and dysfunction of several organs, including the liver, spleen, kidney, and heart (16-19).

There are still not any effective cures for many amyloidosis cases. Therefore, symptomatic treatments and medications are used to prolong life in most patients with amyloidosis and treatments for AA amyloidosis aim to stop the production of SAA (20,21). The use of various drugs that inhibit or suppress the mechanisms that trigger the formation of amyloidosis is currently being developed (22). With the onset of clinical application of glucocorticoids, they have been widely used in the treatment of a wide range of diseases, especially immune-mediated inflammatory disorders (23,24). Methylprednisolone, a class of systemic corticosteroid drugs, is a drug with anti-inflammatory and immunosuppressive effects used as the first line of therapy for the suppression and/or treatment of AA amyloidosis (21,24-28).

In this study, we aimed to investigate the effect of methylprednisolone administration on SAA, TNF-α, IL-1β, and IL-6 levels in mice with experimentally induced AA amyloidosis and to examine its potential therapeutic effects against AA amyloidosis.

2. Materials and Methods

2.1. Animals and Ethical Scope

In this study, 120 female Swiss albino strain mice were used, weighing 23-25 g and 8 weeks old, obtained from Afyon Kocatepe University Laboratory Animal Research Center. Mice were prepared 1 week before the start of the experiment for adaptation to the laboratory environment. Mice were maintained under standard conditions at 24 ± 2 °C with a light-controlled regimen (12 hours light/dark cycle). Mice were fed ad-libitum with the standard mice food (Korkutelim Yem, Türkiye) throughout the study, and drinking water was given regularly. All applications on the animals were performed with the approval of the Local Ethics Committee for Animal Experiments of Afyon Kocatepe University under permit numbers 66-10/140 dated 27.08.2010.

2.2. Preparation of Complete Freund's Adjuvant and Casein Emulsion

Sterile Complete Freund's Adjuvant (CFA) (F5881-10X, Sigma, U.S.A.) and sterile Phosphate Buffered Saline (PBS) were mixed in equal amounts. 8 mg of powdered Casein (218680, Merck, Germany) was added to this mixture and homogenized. CFA-Casein emulsion was administered at a dose of 0.3 ml with the help of a sterile injector to the mice planned to develop amyloidosis (29).

2.3. Experimental Study Design

The mice were divided into 6 equal groups (n: 20) according to the applications to be performed from the beginning of the study to the 8th week. Mice in control groups Group-I (C-IP) and Group-II (C-SQ) were injected respectively intraperitoneally (IP) and subcutaneously (SQ) with 0.3 ml of 0.9% NaCl at 1st and 3rd weeks of the study. Groups planned to develop amyloidosis Group-III (CFA-IP) and Group-IV (CFA-SQ) were injected respectively IP and SQ with 0.3 ml of CFA-Casein emulsion at 1st and 3rd weeks of the study. After the administration of 0.3 ml CFA-Casein emulsion respectively IP and SQ route to the mice in the treatment groups Group-V (CFAM-IP) and Group-VI (CFAM-SQ) at 1st and 3rd weeks of the study, a corticosteroid Methylprednisolone, (Depo-Medrol®, Pfizer, Türkiye) was given intramuscularly (IM) at a dose of 10 mg/kg (29).

All mice were anesthetized with an injection of 10 mg/kg xylazine (Rompun, Bayer, Germany) & 100 mg/kg ketamine (Ketalar, Pfizer, Türkiye) combination by IP route until loss of righting reflex. Blood samples were collected by intracardiac puncture into mice under anesthesia and then animals were euthanized by decapitation. The livers of all animals were removed during systemic necropsy and fixed in 10% buffered formaldehyde for 48 hours. Tissue samples were embedded and solidified into blocks in paraffin after routine histopathological procedures. Paraffin-embedded tissues were cut into 5 μm thickness with a rotary microtome (RM 2155, Leica, Germany) and transferred to the adhesive slides. Slides were stained with Hematoxylin and Eosin (H&E) for routine histopathological examination (30) and alkaline Congo red method for detection of amyloid accumulation (31).

Finally, the slides were examined for histopathologic changes and checked for the presence of amyloid with showing green birefringence in tissue sections and photographed with an imaging system adapted and polarizing filter attached light microscope (ECLIPSE 80i, C-SP 756526, Nikon, Japan). In our study, liver tissue was preferred mainly because it is one of the major sites of deposition of

amyloid fibrils and a major source of synthesis of SAA and pro-inflammatory cytokines.

2.4. Histopathological Analysis

Histology Activity Indexes (HAI) score of liver tissues were examined microscopically on 20x objective magnification in 10 randomly chosen, non-overlapping fields and evaluated semi-quantitatively with a small modification of the Ishak's grading of portal inflammation score evaluation with blind analysis technique. According to this, portal inflammation (inflammatory cell infiltrations) in liver tissues was classified as follows: None, 0; Weak, some of portal areas 1; Weak/moderate, some or all portal areas 2; Moderate, some or all portal areas 3, Moderate/marked, all portal areas 4, Marked, all portal areas 5 (32,33). Amyloid deposition was defined under polarizing microscopy by green birefringence in tissue sections with 20x objective magnification. The findings of Congo red staining were scored as follows: no amyloid deposition (−); weak amyloid deposition (+1); moderate (+2); severe (+3) (12). Positive controls, obtained from known Congo red-positive slides, were always processed in parallel.

2.5. Immunohistochemical Analysis

The liver tissue sections taken from paraffin blocks were used to define the immune reactivity. The slides were deparaffinized with xylene and hydrated with graded alcohols and distilled water. Immunohistochemical examinations were applied with minor modifications of the Streptavidin-Biotin Complex-Peroxidase (SABC-P) method. For this purpose, before the sections taken from the adhesive slides were kept in the 3% hydrogen peroxide (H2O2) (Merck, 88597, Germany) solution for endogenous peroxidase blocking, and heat-induced antigen retrieval was applied to the sections in citrate buffer (pH:6.0) (Merck, 21545, Germany) with microwave for 20 min.

The immune reactivity in the cells was shown by using the anti-SAA (Abcam, Ab2539, UK) (1/160 dilution), anti-TNF-α (Boster, Pa-1079, USA) (1/500 dilution), anti-IL-1β (Santa Cruz, Sc-7884, USA) (1/200 dilution), and anti-IL-6 (Abcam, Ab6672, UK) (1/500 dilution) protein polyclonal antibodies in liver tissues. Protein blocking, secondary antibody (Goat anti-mouse IgG), and enzyme-anti-enzyme complex (SABC-P) applications were achieved by adhering to the conjugation kit (Boster, Sa-2010, USA) application procedure.

3,3'-Diaminobenzidin (DAB) chromogen (Zymed,0 0-2020, USA) was used for the color reaction. Background staining was performed with Mayer hematoxylin (Sigma-Aldrich, 109249, Germany) for one min, and the slides were washed with phosphate-buffered solution (PBS), dehydrated, cleared, and mounted with mounting medium. Positive and negative control slides were used in each application (34). 3 areas in randomly sampled 5 liver sections in each group were randomly investigated under the 20x objective magnification and the immune-positivity intensity (IPI) was scored as follows: none: 0, weak: +1, moderate: +2, severe: +3 (13,35, 36). The presence of immuno-positively stained cells in the slides was examined using the "blind analysis" technique.

2.6. Serological Analysis

The Sandwich ELISA method was applied to blood plasma samples for measuring levels of SAA (CusaBio, CSB-E08590, China), TNF-α (Boster, EK-0527, USA), IL-1β (Boster, EK-0394, USA), and IL-6 (Boster, EK-0411, USA). ELISA kits protocols were administered according to the manufacturer's directions when preparing samples and reagents. Standard solutions and detection curves for each administration were previously made available in the ELISA reader device (SpectraMax Plus 384, USA). Optical density (OD) values of the samples were automatically measured at 450 nanometers (nm) using ELISA reader (37).

2.7. Statistical Evaluation

In determining statistical values of the distribution of amyloid accumulation in tissues according to groups and distribution of serum ELISA values, SPSS 18.0.0 for Windows (Release 18.0.0, Copyright© SPSS Inc, The Apache Software Foundation, 1989-2009) software was used. The Kruskal - Wallis test was used to determine the differences between the groups, and the Nemenyi test was used to reveal the group or groups that made up the difference in cases where there was a difference between the groups as a result of this test. Pearson Correlation test was used to compare the relationship between sera ELISA values of each group. The significance of the correlation coefficients was checked with the "t-test". Statistical analysis of the amyloid formation intensity data in the tissues was also performed with the "Chi-square" test. As a result of statistical evaluations, $P<0.05$ is significant; $P<0.01$ is highly significant; $P<0.001$ was considered very highly significant (38).

3. Results

3.1. Clinical And Macroscopic Results

Clinically, signs of depression (reduction in feed-water consumption and inactivity) and severe abdominal pain findings were observed for 12 hours in all animals treated with CFA. Sudden death was developed in 4 mice in the CFA-IP group and 1 mouse in the CFA-SQ group after the second dose application of CFA. The macroscopic findings of the animals in all groups were evaluated at the end of the 8th week of the experiment. While no macroscopic findings were observed in the mice in the control group, peritoneal-visceral fibrosis and relative enlargement and waxy appearance in the livers in the CFA-IP and CFAM-IP groups; subcutaneous caseous abscesses were seen at the CFA injection site in the CFA-SQ and CFAM-SQ groups.

3.2. Histopathologic Results

H&E staining of liver tissues and inflammatory changes were evaluated in terms of HAI score. HAI scores of all groups were given in Table 1. While different HAI scores were observed in all groups, it was noted that especially in CFA-IP and CFAM-IP, inflammatory cell infiltrations in liver tissues and related HAI scores were higher than in other groups. Inflammatory cell infiltrates were predominantly composed of mononuclear cells (lymphocytes, macrophages and Kupffer cells) and minor amounts of polymorphonuclear leukocytes (neutrophils). It was determined that amyloid deposits in amyloid-positive tissues were stained with H&E in a light pink, eosinophilic character and had an amorphous structure. While necrosis was not observed in the liver parenchyma in the control groups (C-IP and C-SQ), necrosis foci and inflammatory cell infiltrations, hydropic and vacuolar degeneration of hepatocytes, enlargement of sinusoids, and Remark cords irregularities were detected in the CFA-IP, CFA-SQ, CFAM-IP, and CFAM-SQ groups (Figure 1).

Congo red-stained liver tissues were evaluated for amyloid accumulations. Amyloid deposition scores of all groups were given in Table 2. It was noted that especially in CFA-IP and CFAM-IP, amyloid accumulations in liver tissues were more severe than in the other groups. It was determined that amyloid accumulations in tissues showed red-stained fibrillary proteinous character and had an amorphous extracellular structure. Congo red positive stained amyloid deposits were determined to give an apple-green birefringence by microscopic examination under a polarized filter. Amyloid deposits in the liver were found

in the periacinar, periportal, and midzonal regions. It was observed that amyloid deposits were formed primarily in the perivascular areas. It was determined that as the severity of amyloid deposition increased, the Disse (perisinusoidal) spaces became wider by filling with amyloid material, and the Remark cords became irregular (Figure 1).

3.3. Immunohistochemical Results

The immune reactivity in the cells related to anti-SAA, anti-TNF-α, anti-IL-1β, and anti-IL-6 antibodies determined with immunohistochemical staining of liver tissues was evaluated in terms of IPI score. IPI scores of all groups were given in Table 3. Immunohistochemical anti-SAA and anti-TNF-α staining of liver tissues were negative in C-IP and C-SQ groups, and IPI scores were none (0). In immunohistochemical anti-SAA, anti-TNF-α, anti-IL-1β and anti-IL-6 staining, the IPI score was highest, in the CF-IP group at a severe (+3) level; anti-SAA staining in the CFM-IP group, anti-TNF-α, anti-IL-1β and anti-IL-6 staining in the CF-SQ group were at moderate (+2) level; anti-SAA staining in the CF-SQ group, anti-TNF-α staining in the CF-SQ and CFM-SQ groups, anti-IL-β and anti-IL-6 staining in the C-IP and C-SQ groups were at weak (+1) level. Immune-positive anti-SAA, anti-TNF-α, anti-IL-1β and anti-IL-6 staining were observed in the periacinar, periportal, midzonal regions of liver tissues with Kupffer cells and lymphocytes and these stained areas and cells were noted that parallel to amyloid accumulations (Figure 2).

3.4. ELISA Results

For the examinations to be made from blood plasma by ELISA method, 10 samples from each of the mice in the control groups and 16 samples from each of the mice in the amyloidosis-induced groups were evaluated by random sampling. Group averages and differences of plasma SAA, TNF-α, IL-1β, and IL-6 ELISA levels (pg/ml) in blood of animals in experimental groups and statistically significant differences between groups are shown in Table 4. According to the group averages, the highest TNF- α and IL-6 level was found in Group CFA-IP, IL-1β level was found in Group CFA-SQ, and SAA level was found in Group CFAM-IPs. Correlations between plasma SAA, TNF-α, IL-1β, and IL-6 levels were determined by the Pearson correlation test. Accordingly, correlations at $p<0.05$ level were detected between SAA and IL-1β and IL-6; correlations of $p<0.01$ level were observed between SAA and TNF-α and between IL-1β and IL-6 (Table 5).

Table 1: HAI scores* of inflammatory cell infiltrations in livers of experimental groups.

		None (0)	Weak (1)	Weak Moderate (2)	Moderate (3)	Moderate Marked (4)	Marked (5)	Total
C-IP	No / %	16 / 80%	4 / 20%	0 / 0%	0 / 0%	0 / 0%	0 / 0%	20 / 100%
C-SQ	No / %	16 / 80%	4 / 20%	0 / 0%	0 / 0%	0 / 0%	0 / 0%	20 / 100%
CFA-IP	No / %	0 / 0%	0 / 0%	0 / 0%	5 / 31,3%	11 / 68,8%	0 / 0%	16 / 100%
CFA-SQ	No / %	2 / 10,5%	10 / 52,6%	7 / 36,8%	0 / 0%	0 / 0%	0 / 0%	19 / 100%
CFM- IP	No / %	0 / 0%	4 / 20%	5 / 25%	8 / 40%	3 / 15%	0 / 0%	20 / 100%
CFM-SQ	No / %	0 / 0%	5 / 25%	11 / 55%	4 / 20%	0 / 0%	0 / 0%	20 / 100%
Total	No / %	34 / 29,6%	27 / 23,5%	23 / 20%	17 / 14,8%	14 / 12,2%	0 / 0%	115 / 100%

***** Giving the differences in HAI scores between the groups according to the percentile using the Chi-square test.

Table 2: Amyloid deposition severity scores* of liver tissues.

		None (0)	Weak (1)	Moderate (2)	Severe (3)	Total
C-IP	No / %	20 / 100%	0 / 0%	0 / 0%	0 / 0%	20 / 100%
C-SQ	No / %	20 / 100%	0 / 0%	0 / 0%	0 / 0%	20 / 100%
CF-IP	No / %	3 / 18,8%	2 / 12,5%	10 / 62,5%	1 / 6,3%	16 / 100%
CF-SQ	No / %	16 / 84,2%	3 / 15,8%	0 / 0%	0 / 0%	19 / 100%
CFM- IP	No / %	15 / 75%	3 / 15%	2 / 10%	0 / 0%	20 / 100%
CFM-SQ	No / %	18 / 90%	2 / 10%	0 / 0%	0 / 0%	20 / 100%
Total	No / %	92 / 80%	10 / 8,7%	12 / 10,4%	1 / 9%	115 / 100%

***** Giving the differences in Amyloid deposition severity scores between the groups according to the percentile using the Chi-square test.

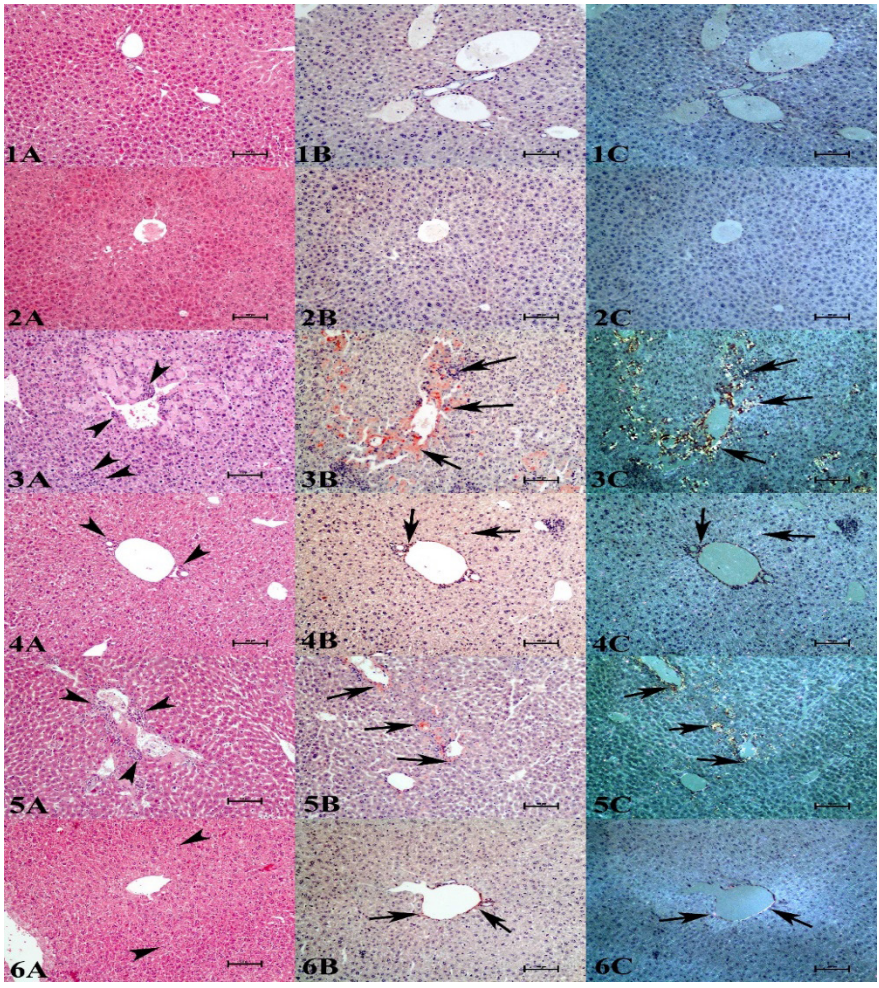

Figure 1: Histopathology of liver tissues. A: H&E staining; B: Congo red staining; C: Polarized image of Congo red staining (x100 magnifications). Normal histology of the liver tissues and absent amyloid staining with apple-green birefringence on polarized image in the control groups (C-IP and C-SQ respectively 1A, B, C and 2A, B, C). Moderate/Marked inflammatory cell infiltrations are shown with arrowheads in CFA-IP and CFAM-IP groups respectively (3A and 5A). Weak inflammatory cell infiltrations are shown with arrowheads in CFA-SQ and CFAM-SQ groups respectively (4A and 6A). Severe and moderate amyloid depositions are shown with arrows in CFA-IP and CFAM-IP groups respectively, light pink (3A and 5A), red stained (3B and 5B) and apple-green birefringence (3C and 5C). Weak amyloid depositions are shown with arrows in CFA-SQ and CFAM-SQ groups respectively, light pink (4A and 6A), red stained (4B and 6B) and apple-green birefringence (4C and 6C).

Table 3: IPI scores of IHC SAA, TNF-α, IL-1β, and IL-6 staining of liver tissues.

			None (0)	Weak (1)	Moderate (2)	Severe (3)	Total
SAA	C-IP	No / %	5 / 100%	0 / 0%	0 / 0%	0 / 0%	5 / 100%
	C-SQ	No / %	5 / 100%	0 / 0%	0 / 0%	0 / 0%	5 / 100%
	CF-IP	No / %	0 / 0%	0 / 0%	1 / 20%	4 / 80%	5 / 100%
	CF-SQ	No / %	0 / 0%	5 / 100%	0 / 0%	0 / 0%	5 / 100%
	CFM- IP	No / %	0 / 0%	1 / 20%	3 / 60%	1 / 20%	5 / 100%
	CFM-SQ	No / %	0 / 0%	4 / 80%	1 / 20%	0 / 0%	5 / 100%
Total		No / %	**10 / 33,3%**	**10 / 33,3%**	**5 / 16,7%**	**5 / 16,7%**	**30 / 100%**
TNF-α	C-IP	No / %	0 / 0%	5 / 0%	0 / 0%	0 / 0%	5 / 100%
	C-SQ	No / %	0 / 0%	5 / 0%	0 / 0%	0 / 0%	5 / 100%
	CF-IP	No / %	0 / 0%	0 / 0%	0 / 0%	5 / 100%	5 / 100%
	CF-SQ	No / %	0 / 0%	2 / 40%	3 / 60%	0 / 0%	5 / 100%
	CFM- IP	No / %	0 / 0%	1 / 20%	2 / 40%	2 / 40%	5 / 100%
	CFM-SQ	No / %	0 / 0%	2 / 40%	3 / 60%	0 / 0%	5 / 100%
Total		No / %	**0 / 0%**	**15 / 50%**	**8 / 26,7%**	**7 / 23,3%**	**30 / 100%**
IL-1β	C-IP	No / %	1 / 20%	4 / 80%	0 / 0%	0 / 0%	5 / 100%
	C-SQ	No / %	1 / 20%	4 / 80%	0 / 0%	0 / 0%	5 / 100%
	CF-IP	No / %	0 / 0%	0 / 0%	0 / 0%	5 / 100%	5 / 100%
	CF-SQ	No / %	0 / 0%	1 / 20%	4 / 80%	0 / 0%	5 / 100%
	CFM- IP	No / %	0 / 0%	2 / 40%	3 / 60%	0 / 0%	5 / 100%
	CFM-SQ	No / %	0 / 0%	3 / 60%	2 / 40%	0 / 0%	5 / 100%
Total		No / %	**2 / 6,7%**	**14 / 46,7%**	**9 / 30%**	**5 / 16,7%**	**30 / 100%**
IL-6	C-IP	No / %	0 / 0%	5 / 100%	0 / 0%	0 / 0%	5 / 100%
	C-SQ	No / %	0 / 0%	5 / 100%	0 / 0%	0 / 0%	5 / 100%
	CF-IP	No / %	0 / 0%	0 / 0%	0 / 0%	5 / 100%	5 / 100%
	CF-SQ	No / %	0 / 0%	0 / 0%	5 / 100,0%	0 / 0%	5 / 100%
	CFM- IP	No / %	0 / 0%	0 / 0%	4 / 80%	1 / 20%	5 / 100%
	CFM-SQ	No / %	0 / 0%	2 / 40%	3 / 60%	0 / 0%	5 / 100%
Total		No / %	**0 / 0%**	**12 / 40%**	**12 / 40%**	**6 / 20%**	**30 / 100%**

*Giving the differences in IPI scores between the groups according to the percentile using the Chi-square test.

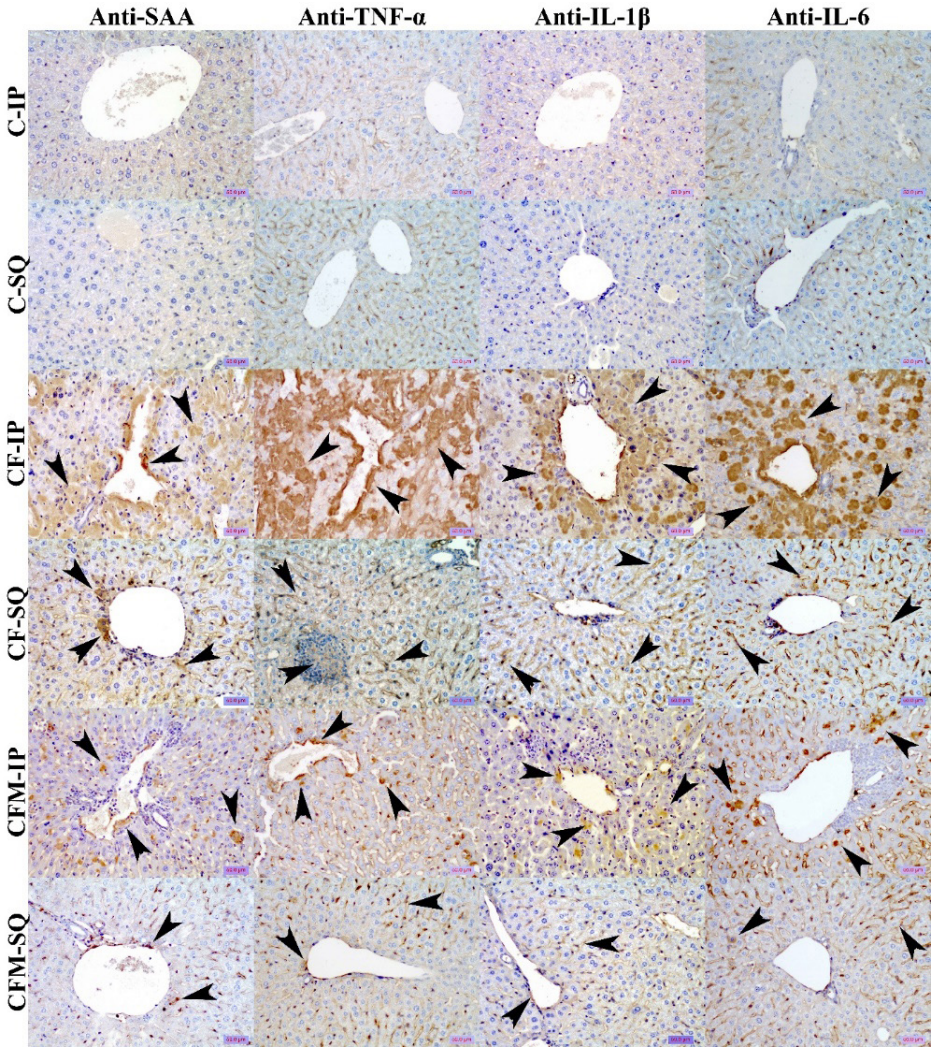

Figure 2: Anti-SAA, Anti-TNF-α, Anti-IL-1β, and Anti-IL-6 immune-reactivity of the liver tissues of experimental groups. Arrowheads show immune-positive stained areas. IPI scores are seen: None (0) in the control groups (C-IP and C-SQ); Severe (+3) in the CF-IP group; Moderate (+2) in the CF-SQ and CFM-IP groups; Weak (+1) in the CFM-SQ group. DAB chromogen was used show to immune positivity and background stained with Mayer's Hematoxylin. Immunohistochemical SABC-P staining method, X200 magnifications.

Table 4: Group averages and differences of plasma SAA, TNF-α, IL-1β and IL-6 ELISA levels (pq/ml) in experimental groups.

Groups	SAA	TNF-α	IL-1β	IL-6
C-IP	19.04±5.4	2.71±2.98	18±20.25	8.16±9.52
C-SQ	23.89±5.13	7.72±4.78	10.3±1.83	6.74±4.86
CFA-IP	25.81±5.69	686.35±396.94***,###	18.63±7.92#	71.66±48.54***, ###
CFA-SQ	21.14±6.97	67.83±45.67***, #	19.5±13.16	26.42±19.97*, #
CFAM-IP	38.93±12.49***,$$	37.84±52.81*,+++	14.44±4.41	12.57±15.74+++
CFAM-SQ	15.05±5.75#,++,&&&	25.1±10.33*, ++	13.88±7.92	39.03±26.27**, ###, &

* P<0.05, ** P<0.01, *** P<0.001 compared to group C-IP; # P<0.05, ### P<0.001 compared to group C-SQ; ++ P<0.01**,** +++ P<0.001 compared to group CFA-IP; **$$** P<0.01 compared to group CFA-SQ; **&** P<0.05, **&&&** P<0.001 are statistically significant at different levels when compared with group CFAM-IP (Kruskal-Wallis and Nemenyi Comparison of Different Data Groups Test). Data are shown as mean and standard deviation.

Table 5: Correlation relationships between plasma SAA, TNF-α, IL-1β, and IL-6 levels.

		No	SAA	TNF-α	IL-1β	IL-6
No	Pearson Correlation	1	,043	,054	-,020	,192
	Sig. (2-tailed)		,783	,730	,894	,217
	n	45	44	44	45	43
SAA	Pearson Correlation			,541**	,331*	,312*
	Sig. (2-tailed)			,000	,028	,042
	n			44	44	43
TNF-α	Pearson Correlation				,254	,209
	Sig. (2-tailed)				,096	,178
	n				44	43
IL-1β	Pearson Correlation					,451##
	Sig. (2-tailed)					,002
	n					43

* P<0.05 and ** P<0.01, compared with SAA; ## P<0.01, indicates statistical significance at different levels when compared with IL-1β (Pearson correlation test performed as a result of the Kruskal-Wallis and Nemenyi Comparison of Different Data Groups test). n: number of samples; Sig. (2-tailed): Significance level, two-way.

4. Discussion

AA Amyloidosis is a progressive, destructive disease characterized by organ dysfunction caused by extracellular and intracellular deposition of N-terminal fragments of SAA synthesized in certain organs, especially the liver, in the form of insoluble amyloid fibrils in the β-sheet structure as a result of disruption of normal cell and organ physiology (1,18,22, 39-41). Amyloid A deposits occur as a consequence of chronic inflammatory disease due to a prolonged and persistent supersaturated state of SAA, an apolipoprotein of high-density lipoproteins, an acute phase reactant (12,42,43). In systemic AA amyloidosis, the most common type of amyloidosis in mammals and birds, the source of inflammatory stimuli is associated with a wide variety of chronic diseases, neoplasms, and idiopathic causes (15,44,45).

In experimental animals such as mice and guinea pigs, AA amyloidosis can also be induced experimentally by prolonged administration of inflammatory stimuli (41,43). In mice, AA amyloid deposition can be experimentally induced by multiple subcutaneous or intraperitoneal injections of preformed AA fibrils, amyloid enhancing factor (AEF) (46), silver nitrate (AgNO3) (47), casein or lipopolysaccharide (LPS) (Complete Freund's Adjuvant), thereby significantly increasing the level of SAA in plasma and maintaining this high level (48). In AA amyloidosis-induced animals, clinical changes in the liver, spleen, and kidney enzymes and relative enlargement of these organs according to the severity of amyloid accumulation were detected (48-50). In addition, it has been determined that ruptures and softening may occur in the spleen and liver due to amyloid deposition and the hardening of these organs associated with chronic tissue damage (49). In our study, it was observed that the clinical findings and macroscopic lesions were more severe in mice in the experimental and treatment groups in which amyloidosis was induced by the intraperitoneal route compared to subcutaneously amyloidosis-induced mice. According to the necropsy findings, it was determined that amyloidosis induced by the intraperitoneal route was very severe compared to the subcutaneous procedure and was similar to the literature data.

In mice, no amyloid deposits were detected at day 16 after AgNO3 administration alone, whereas in experimental AA amyloidosis induced by AEF and AgNO3, amyloid deposits have been detected as early as day 2 after injection in spleen. The starting point of amyloidogenesis has been reported to be almost 2 days before amyloid deposits could be demonstrated in cross-polarized light by Congo red staining. Amyloid deposits were observed in the liver of 11 of 17

mice with splenic amyloidosis on day 4 and in all mice on day 16 after AEF and AgNO3 injection. In addition, it has been reported that amyloid deposits did not develop in the liver in mice without splenic amyloidosis (47,51). In our study, five weeks after the second injection of CFA-casein emulsion, it was observed that induction of amyloidosis by the IP route caused amyloid deposits at levels ranging from mild to severe and at higher rates compared to the SQ route. Our findings confirm that the liver is one of the main target sites of amyloid fibril deposits.

The deposition of AA amyloid fibrils, which usually starts in the spleen and liver, may eventually spread to the kidney, adrenal gland, gastrointestinal tract, peripheral nervous system, respiratory system, and skin tissue (6,52). It has been reported that the amounts of AA amyloid fibrils accumulated in organs were spleen, liver, kidney, and intestine from the highest to the lowest level, respectively (13). Macroscopically, affected tissues in systemic amyloidosis are characteristically enlarged, firm and waxy in appearance. (18) In addition, it has been reported that the amount of amyloid fibril accumulation in mice with amyloidosis causes enlargement of liver and spleen and an increase in abdominal fat (53). Hepatomegaly with splenomegaly may be seen in humans with amyloidosis (7,54). In this study, the highest amount of amyloid fibril deposits in the liver tissues were found after IP injection of CFA-casein emulsion and the findings observed at necropsy were similar to those of the researchers. Intraperitoneal injection of CFA-Casein emulsion is a more successful method to induce AA amyloidosis in mice than subcutaneous injection.

In one study, it was found that the rate of amyloidosis formation was 80% after IP injection of CFA-casein emulsion in mice fed with high fat and cholesterol-containing feeds (53); while in another study, it was reported that the rate of amyloidosis formation was 100% as a result of casein injection alone (55). It was also found that the rate of experimental amyloid arthropathy formation in brown layer hens with CFA-casein emulsion injection was 100% (27). In this study, we found the highest rate of amyloid accumulation in liver tissue (81.3%) in the CFA-IP group and this data is in parallel with the literature data. Considering the differences in the amyloid deposition rates in the liver tissues of the experimental groups, our study confirms the finding of Cui, Dan et al. (2008) that liver tissue is one of the main deposition sites of AA amyloid fibrils (56). Mice treated with CFA-casein emulsion alone showed a significant increase in amyloid deposition in the liver compared to the other groups. The relative enlargement and waxy appearance in the livers of mice with amyloid deposition were consistent with the macroscopy of AA amyloidosis.

Inflammation can alter the histomorphology of tissues in which amyloid deposits form and induce the progression of AA amyloidosis (11). Histological examinations of AA amyloidosis in cattle show that severe AA amyloid deposits can occur in various organs throughout the body (41). Symptomatic dysfunction due to amyloidosis is rare and presents late clinically; therefore, liver biopsy is the gold standard for diagnosis. Hepatic amyloid deposits spread arteriolar, and/or capsular, sinusoidal, and portal areas (57). It has been claimed that amyloid deposits in liver tissue are mainly formed in the portal area and spread along the sinusoids over time (47). Microscopically, amyloid deposits as extracellular proteinaceous deposits that are eosinophilic and glassy. Congo red stain shows a red to orange appearance to amyloid, with apple-green birefringence under polarized light. Amyloid fibrils can be also confirmed using electron microscopy to defined 7.5- to 10-nm diameter unbranched fibrils in affected tissue (18,22). In AA amyloidosis induced by CFA-casein emulsion injection in mice, we supposed that inflammatory cell reactions play a very important role in the pathogenesis of AA amyloidosis due to the increase in mononuclear and polymorphonuclear cell infiltrations, which were parallel to the increase in HAI scores we detected in liver tissues positive for amyloid deposition with H&E staining. The amorphous eosinophilic structures stained with H&E were stained with Congo red, and the apple green color and double reflection observed in the polarized filter microscope proved that there were amyloid deposits and these findings were similar to the literature data. However, the finding that the amyloid deposits in our study occurred primarily in the perivascular areas and that the perisinusoidal spaces were filled with amyloid deposits in samples with more severe deposition is not consistent with the findings of Nyström et al. (47).

In human AA amyloidosis, plasma SAA concentration is an important factor in determining amyloid deposition (58). Furthermore, proteins (even synthetic peptides) with different amino acid sequences from murine SAA have been observed to exhibit amyloidogenic fibril-forming activity (59). Most of circulating SAA links to its main plasma carrier, high-density lipoprotein (HDL), and thereby reorganize routes HDL transport and promotes lipid clearance from the locations of injury and lipid recycling for tissue reparation. Linking to HDL stabilizes the α-helical formation in SAA and keeps it from proteolysis and misfolding. The misfolding of SAA underlies AA amyloidosis formation mechanism. Although linking to the lipid surface promotes normal functions of SAA in lipid transport, SAA mutual effects with cell membranes may also modulate pathologic effects in AA amyloidosis (15,18,40,60,61). The SAA genotype and the total period of inflammatory processes significantly increase

the risk of AA amyloidosis (62). In our study, the highest plasma SAA level was seen in the groups in which CFA-casein emulsion was administered via the IP route. In amyloidosis induced in mice after 2 doses of IP administration of CFA-Casein emulsion, a period of 5 weeks is sufficient for SAA concentration to increase to high levels and amyloid deposition to occur in liver tissue. It is also observed that there is a parallelism between the increasing SAA level in the blood and the severity of amyloid deposition.

SAA is not a single type of protein; it is a family of proteins encoded by a highly conserved gene family that includes up to 5 SAA genes in various species (18). There are four SAA gene isoforms in humans; while SAA1 and SAA2 encode the acute phase, SAA3 is a distinct pseudogene and SAA4 encodes a constitutively expressed isoform. SAA is produced by hepatocytes and other cells (Kupffer liver cells, fibroblasts, vascular endothelial cells, and reticuloendothelial cells) under cytokine control and secreted into plasma where it associates with lipoproteins (19,60). In mice, ApoA-II is the serum precursor of amyloid fibrils (AApoAII) in age-mediated systemic amyloidosis (AApoAII amyloidosis). It has been reported that mouse SAA, ApoA-I, and ApoA-II interact with each other during AA and AApoAII amyloidosis. It has been observed that during acute phase response, increased SAA links to HDL and decreases levels of ApoA-I and ApoA-II, leading to alteration of HDL particle size and ApoA-II has been shown to trigger inflammation and increase cytokines and macrophages. When the increased level of ApoA-I was compared with ApoA-II, ApoA-II was found to be a more potent modulator of apolipoprotein stabilization and lipoprotein metabolism (11). In contrast to AA amyloidosis-susceptible mouse strains, which co-synthesize SAA type 1 and 2 isoforms encoded by the SAA1 and SAA2 genes, respectively, in response to inflammatory stimulation from the liver, only a single SAA isoform, SAA2.2, is synthesized in CE/J strain mice, which are resistant to AA amyloidosis (63). It is observed that Swiss albino mice used in our study are susceptible to amyloidosis. In addition, the presence of positive SAA staining by IHC observed in Kupffer cells and hepatocytes in mice developing AA amyloidosis supports the literature data. However, more comprehensive molecular and genetic studies are needed to prove the relationship between this finding and SAA gene isoforms in Swiss albino mice.

IHC is the most common technique used to determine amyloid fibril type (7,14). For example, it was experimentally demonstrated by IHC that bovine AA fibrils can seed murine AA fibrils. (13) In a long-term kinetic study, AA

amyloidosis was induced in mice using homologous AA fibrils extracted from amyloid-containing murine spleen tissue and revealed that peak levels of AA accumulation followed the natural clearance of AA deposits from the body. Pro- and anti-inflammatory cytokine release has been observed during AA deposition and elimination, respectively (13,41,64). After injection of amyloid fibrils, AA amyloid deposition has been observed in the livers and spleens of wild-type and Apoa2 knock-down mice. As the time elapsed since injection increased, AA amyloid deposition has been shown to expand from the liver and spleen to the stomach, intestine, lung, and kidney (11). Immunohistochemical staining of the liver with anti-SAA antibody 24 hours after amyloidogenic stimulation showed that the reaction products appeared linearly at the sinusoidal margins. After colchicine treatment, reaction products were mainly seen in hepatocytes at the periphery of the lobules, whereas after triptolide treatment, reaction products were not detected in the liver (65). Since the liver is the main production site of SAA and an important deposition site of amyloid fibrils, the liver was considered the target tissue in our study. Immunohistochemically, we obtained that the severity of amyloid deposition, TNF-α, IL-1β, and IL-6 levels were significantly higher in the CFA-IP group compared to the other groups. This finding indicated that IP administration of CFA-Casein emulsion resulted in a stronger inflammatory process and its amyloid-inducing potential was quite successful. Based on our immunohistochemical findings, SAA, TNF-α, IL-1β, and IL-6 play an important role in the formation of amyloidosis. In the pathogenesis of AA amyloidosis, it is evident that there is a directly proportional relationship between the severity of amyloid fibril deposition in tissues and liver SAA and pro-inflammatory cytokine IHC staining levels. These findings were also consistent with the findings of studies reporting increased levels of SAA, TNF-α, IL-1β, and IL-6 in experimental amyloidosis in animals. Furthermore, we found a significant decrease in IHC staining levels of SAA and pro-inflammatory cytokines in hepatic lobules after methylprednisolone treatment.

Macrophages and monocytes activated during inflammation can partially degrade SAA and trigger AA amyloid fibril formation. It has also been reported that macrophages can phagocytize amyloid fibrils (58,66,67). Extracellular amyloid fibrils are readily identified by the host's cellular immune system (neutrophils or macrophages). These immune cells have receptors that recognize and bind to amyloid fibrils and their intermediates. They thus activate a signaling cascade that leads to the production of pro-inflammatory cytokines. It is now known that the expression of this particular amyloidogenic protein

is regulated by IL-6, a pro-inflammatory cytokine, leading to increased levels of SAA (43). It has been suggested that cytokines and macrophages may have an immunologic relationship with amyloid deposition and elimination (13). In experimentally induced models of amyloidosis in mice and chickens, the synthesis of IL-1 and IL-6 has been shown to be significantly and to varying degrees increased (12,45,68). Immunoreactivity to anti-SAA, anti-TNF-α, anti-IL-1β and anti-IL-6 antibodies determined by IHC staining of liver tissues also revealed an association between amyloid fibril deposition and pro-inflammatory cytokines and SAA. Hepatocytes, Kupffer cells and lymphocytes are involved in the pathogenesis of pro-inflammatory cytokine and SAA-associated amyloid fibril formation.

It has been reported that treatment of patients with AA amyloidosis can be successful when the SAA concentration is kept below 10 mg/l (7,8,63). In a transgenic mouse model, it has been shown that the time and extent of amyloid deposition is determined by plasma SAA concentration and that amyloid deposition occurs independently of inflammation (69). Some studies have reported a high degree of variation in the amount of AA deposition, serum cytokine profiles, and different parameters depending on the experimental amyloidosis model. It has been suggested that this may be due to the induction of amyloidosis by heterologous AA fibrils (13). This may be due to differences in individual susceptibility of mice in relation to the amyloidosis induction model. In our study, plasma SAA levels were significantly higher in the groups in which amyloidosis was formed by the IP route. At the same time, although it is certain that the increase in SAA levels is associated with an increase in pro-inflammatory cytokine levels such as TNF-α, IL-1β, and IL-6, it is controversial whether the increase in SAA levels is associated with an increase in pro-inflammatory cytokine levels or whether the increase in pro-inflammatory levels primarily contributes to the increase in SAA levels. Because in our study, when plasma SAA and pro-inflammatory cytokine levels were compared between the amyloidosis-induced groups and the methylprednisolone-treated groups, no direct correlation was observed unlike IHC findings in the liver.

The synthesis of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α related to effect of AA amyloidosis development mechanism involved in chronic inflammation stimulate the liver cells and upregulate the production of SAA, resulting in SAA being maintained at high levels (12,18,41,59,70). IL-1β can inhibit SAA degradation. This situation provokes to polymerization into amyloid fibrils resulting in the formation of amyloidogenic SAA fragments.

Some experiments demonstrate that TNF- α and IL-1β potentiate each other's synthesizing and up-regulate IL-6. SAA is affected by primarily to IL-1, but high stimulation requires the synergistic action of both IL-1 and IL-6. Although IL-6 can stimulate IL-1 and TNF-α production and synergistically cause SAA synthesis (8, 12,43,70,71). Mice inoculated with AA fibrils to induce experimental amyloidosis have been shown to rapidly increase and then decrease IL-6 and the significance of IL-6 in the induction of AA amyloidosis has been proven (13,43,72). There is a positive correlation between the severity of amyloid deposition and serum SAA, IL-1β, and TNF-α levels both in tissues and serum. Furthermore, IL-1β plays an important role in poultry AA amyloidosis together with TNF-α (45). In our study, statistically significant differences between plasma SAA, TNF-α, IL-1β, and IL-6 levels of control and amyloid-induced groups indicate that pro-inflammatory cytokines and SAA synthesis stimulate each other. The statistically demonstrated correlation between plasma SAA, TNF-α, IL-1β, and IL-6 levels suggest that these parameters are important factors involved in the formation of AA amyloidosis. In AA amyloidosis, changes in plasma SAA levels are associated with changes in TNF-α, IL-1β, and IL-6 levels, while changes in IL-1β and IL-6 levels are associated with each other.

The fact that amyloid deposits have the potential to affect almost all organ systems means that the clinical features of systemic amyloidosis are diverse and rarely result from a single type of amyloidosis, leading to difficulties and delays in diagnosis. Determining the type of amyloid fibril is crucial for the choice of treatment modality (14). The formation of amyloid fibrils in systemic AA amyloidosis has already been investigated in many aspects, but the whole cellular mechanism has not yet been fully elucidated, especially the question of whether amyloid formation starts intracellularly is still controversial (8).

Once amyloidosis has occurred, the best therapeutic approach is to stop the ongoing inflammatory activity to prevent further amyloid formation. Recent advances in the understanding of the molecular pathogenesis of AA amyloidosis have led to different therapeutic options applicable to the amyloidogenic process. These include stabilizers of the native structure of the amyloidogenic precursor, amyloid fibril degraders, fibrillogenesis suppressors and protectors of amyloid clearance. Innovative pharmacological strategies such as structure-based drug design, gene therapy and immunotherapy, as well as the repurposing of old, safe drugs with newly recognized anti-amyloid properties, are currently being studied and are already evolving clinically (20,22,26). In addition to proving the presence of amyloid deposits, it is crucial to determine the type of amyloid fibril

present to guide treatment (7). Clinically, many drugs can be very effective in the treatment of AL and AA amyloidosis but are often not well tolerated because they can cause impairment of organ function. The assessment of response in patients with amyloidosis involves a combined evaluation of the effects of treatment, firstly, on the suppression of amyloid precursor protein synthesis and secondly, on the function of amyloidotic organs. Decreasing the production and level of SAA by treating the inflammatory disease that mainly causes AA amyloidosis is key to managing AA amyloidosis, but this is not yet possible for some types of amyloidosis (20,21,26). This may have more successful outcomes as much more powerful anti-inflammatory drugs are now available for treatment (62). Compared to the amyloidosis-induced CFA-IP and SQ groups, we found that in CFAM-IP and SQ groups treated with methylprednisolone, plasma TNF-α, IL-1β, and IL-6 levels were suppressed as well as TNF-α, IL-1β, and IL-6 IHC staining levels in liver tissues were partially suppressed. The statistically significant correlation observed between SAA and TNF-α, IL-1β, and IL-6 in our study reveals the effects of these pro-inflammatory cytokines on the pathogenesis of AA amyloidosis.

The efficacy of AA amyloidosis treatments with corticosteroids is still controversial (39,43). Corticosteroids can suppress the increase of acute phase reactions, including C-reactive protein (CRP) and SAA synthesis. In human hepatocyte cultures, some corticosteroids were found to stimulate SAA production but not CRP production (73). Many studies have shown that SAA is sensitive to change, reaching much higher levels than CRP but decreasing more rapidly, and therefore may accurately reflect disease activity (74). Therefore, it is advisable to assess SAA levels rather than CRP levels, especially when corticosteroids are used. Treatment of patients with AA amyloidosis with cytotoxic drugs alone or in combination with prednisolone, a synthetic glucocorticoid, and cortisol derivative, is therefore considered appropriate (25). Some studies suggest a synergistic effect of glucocorticoids and cytokines in the induction of SAA synthesis (39,43). The severity of amyloid deposition in the amyloidosis-induced groups was significantly higher than in the methylprednisolone-treated groups. This finding suggests that methylprednisolone exerts its effect primarily by inhibiting fibrillogenesis rather than suppressing SAA synthesis. In our study, SAA levels in the methylprednisolone-treated groups were relatively higher but pro-inflammatory cytokine levels were lower compared to the other groups, suggesting that the effect of methylprednisolone is probably primarily mediated by suppression of the synthesis of pro-inflammatory cytokines.

Since the effect of immunosuppressants may take weeks or months to become apparent, steroids in addition to immunosuppressants are recommended to achieve an immediate reduction in the acute phase response and especially in SAA synthesis (21,75). In humans, SAA concentrations of less than 4 mg/L are associated with a good prognosis of AA amyloidosis. The choice of treatment method should be based on the nature of the underlying problem. Colchicine (65), a glucocorticoid used for familial Mediterranean fever; TNF inhibitors tocilizumab (71,76), etanercept, infliximab, and adalimumab; and biological therapeutics such as anakinra or canakinumab, which are IL-1 blockers (70), are also used in the treatment of amyloidosis. Therefore, it has been suggested that inhibition of the release and activity of IL-1 is the best effective way to prevent and curing AA amyloidosis. In patients with idiopathic inflammatory AA amyloidosis, cytokine receptor blockers may respond to specific inhibition of interleukin IL-1 or IL-6 (14,15,21,26) .

If corticosteroids are used to treat AA amyloidosis, monitoring of SAA levels is particularly recommended. Treatment of patients with AA amyloidosis with cytostatic drugs alone or in combination with prednisolone is also a good option (17). Prednisolone significantly inhibited IL-l production by LPS-stimulated monocytes but increased SAA production synthesized by hepatocytes (77). The combination of dexamethasone with bortezomib and cyclophosphamide has been reported to achieve a response rate of more than 90% and a complete response rate of 60% in patients who had not achieved adequate results in previous treatment (7,76). For example, treatment with oral melphalan and high-dose dexamethasone has shown good results in the prognosis and overall survival of patients with amyloidosis >7 years (22). Prednisolone is also used in AA amyloidosis patients with protein-losing enteropathy (75). AA amyloidosis patients with the SAA1.3 allele have been shown to be sensitive to prednisolone and cyclophosphamide combination therapy. (78) Although many corticosteroid drugs have been used to treat AA amyloidosis, data on the use of methylprednisolone in mammals are minimal. Furthermore, in our study, the suppressive effect of methylprednisolone on pro-inflammatory cytokine levels and amyloid deposits was demonstrated both serologically and histopathologically.

Methylprednisolone, a synthetic glucocorticoid, acts by suppressing the synthesis of pro-inflammatory cytokines by promoting the expression of anti-inflammatory gene products while blocking the function of the receptor complex pro-inflammatory genes and transcription factors. Thus, methylprednisolone

demonstrates efficacy for the suppression and/or treatment of AA amyloidosis (28). Methylprednisolone at a dose of 1 g given orally in pulse therapy in patients with gastrointestinal AA amyloidosis (79) and at a dose of 60 mg/day in a patient with AA amyloidosis associated with ulcerative colitis have been shown to be successful (80). In the long-term follow-up of AA amyloidosis in a patient with rheumatoid arthritis, methylprednisolone administration at a dose of 2 mg/day was reported to be successful in the treatment of amyloidosis and in reducing serum SAA and IL-6 levels (25). Furthermore, in the acute and late phase of the amyloid arthropathy model in chickens, it was found that the increase in serum SAA, IL-1β, IL-6, and TNF-α levels were parallel and methylprednisolone had a suppressive effect in decreasing these levels (27,68). In our study, it is clear that plasma TNF-α, IL-1β and IL-6 levels and tissue IHC staining scores were significantly decreased due to methylprednisolone administration at a dose of 10 mg/kg. In parallel with the decrease in pro-inflammatory cytokine levels, the severity of amyloid deposition in liver tissue also decreased. Therefore, pro-inflammatory cytokine levels are directly proportional to SAA levels and amyloid fibril deposition severity.

5. Conclusion

Intraperitoneal administration of CFA-Casein emulsion is more effective than subcutaneous route in forming AA amyloidosis in liver tissue and in increasing SAA, TNF-α, IL-1β, and IL-6 levels serologically and IHC. There is a parallelism between increased SAA levels in blood and tissue, the severity of amyloid fibril deposition, and TNF-α, IL-1β, and IL-6 levels. Methylprednisolone administration at a dose of 10 mg/kg decreases TNF-α, IL-1β, and IL-6 levels on the one hand, and on the other hand, it has a positive effect on the inhibition of amyloid fibril formation in liver tissue, while it seems to be insufficient in decreasing SAA levels.

In conclusion, SAA, TNF-α, IL-1β, and IL-6 play an important role in the formation of AA amyloidosis. On the other hand, there is a significant correlation between SAA and pro-inflammatory cytokines and between IL-1β and IL-6. In addition, repeated and different doses of methylprednisolone and more comprehensive histopathologic, immunohistochemical and serologic investigations should be performed to suppress AA amyloidosis with methylprednisolone, to elucidate the mechanism of amyloid fibril formation and degradation and to determine the relationship between SAA, TNF-α, IL-1β and IL-6 levels.

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CHAPTER II

CANINE DEMODICOSIS

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1. Introduction

A parasitic skin disease known as demodicosis is frequently found in dogs and is characterized by an excessive number of Demodex mites on the skin's surface and hair follicles (1). These mites are obligate parasites and are part of the skin flora in small numbers of healthy dogs. It can be transmitted directly from the mother to her offspring through close contact 2-3 days after birth (2-5). These mites can be isolated from the hair follicles of healthy dogs, especially on the face and nose area. These mites spend their entire life cycle within their hosts and feed on skin cells, sebum, and epidermal debris (6). However, they are found in much greater numbers in the skin of sick animals (7,8). It is known that many factors affect the increase in the number of mites. In generalized demodicosis in young dogs, any immune-related changes play a crucial part in the pathogenesis. In older dogs, demodicosis is observed due to immunity or immunosuppressive drugs used (6,8-10). On the other hand, demodicosis occurring in young dogs may be genetic in origin and more than one gene may play a role in the development of the disease. Additionally, there are other unknown factors involved (11).

Alopecia, comedones, follicular casts, papules, and pustules are signs of canine demodicosis (7,12). The level of mite reproduction determines how serious the clinical signs are. Initial symptoms might include a non-inflammatory hypotrichosis/alopecia and/or an inflammatory dermatitis with accompanying hypotrichosis/alopecia, moderate erythema, comedone development, scaling, and inflammatory dermatitis. In dogs with severe demodicosis; secondary bacterial infections may also occur along with systemic clinical symptoms. In cases of secondary infection, itching may also occur. Depending on the localization and generalization of the disease, the lesions can be seen on the face, extremities and the entire body surface (7,13). The lesions might range from being localized or multifocal to aggregating and affecting significant areas of the body. It is possible for hair follicular ostia to exhibit follicular plugging, dilatation, and hyperpigmentation; when this occurs, it is a clinical sign that the condition is present (8,11).

1.1. Etiology

There are three recognized canine Demodex mites. These are *Demodex canis* (*D. canis*), *Demodex injai* (*D. injai*) and *Demodex cornei* (*D. cornei*). (13-15) The most common demodectic mite of dogs is *D. canis* (Figure 1). In a study conducted in 2012, the phylogenetic relationship of these three species

was investigated and it was determined that there was a 76.7% genetic similarity between *D. canis* and *D. injai*. Mitochondrial rRNA sequences of *D. cornei* were determined to be 99.5% similar to *D. canis* (16). Some researchers have stated that the short-stemmed mite called *D. cornei* is a morphological variant of *D. canis*(17,18). Taxonomic study revealed that the short-bodied mite is a different species of dog (15).

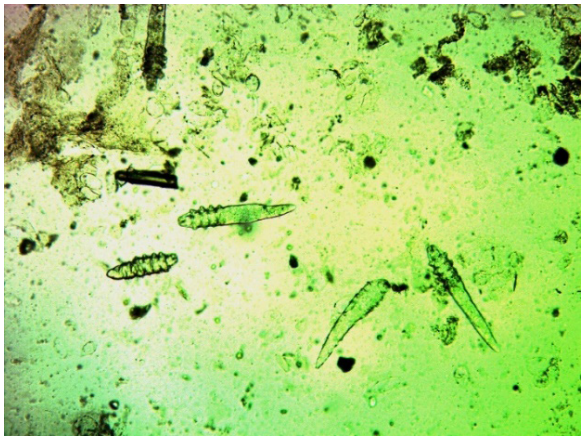

Figure 1. Skin scraping reveals *D. canis* mites (x10)

1.2. Clinical Forms of Demodicosis

There are two types of demodicosis: localized and generalized, with either a juvenile onset form or an adult-onset form. Depending on the form of the disease, there are various demodicosis prognoses and treatment choices (11).

1.2.1. Localized Demodicosis

The body's four or fewer focus points, frequently the face, neck, and forelegs, are involved in the localized form (Figure 2). The lesion diameter is below ≤2.5 cm. In juvenile dogs; localised demodicosis presents as patches of hair loss and red inflamed skin These patches often occur around the face, head and feet. Itching is not usually seen at the beginning of the disease, but it may develop later as a result of pyoderma due to secondary bacterial infections. Localized demodicosis has a good prognosis. It may resolve spontaneously in six to eight weeks without treatment. In young dogs; mild and localized forms of demodicosis may not require treatment and may resolve spontaneously as the animal ages. If treatment is not given in such cases, it should be closely

monitored. However, topical treatment with shampoos or gels containing lime sulfur or benzoyl peroxide may also be recommended (6,19).

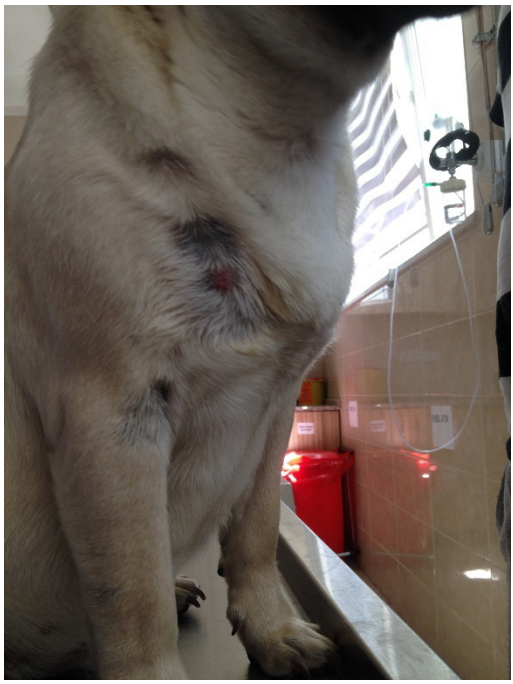

Figure 2. A 5-month-old Anatolian shepherd dog with localized demodicosis showing several circular areas of alopecia and hyperemia on the chest area

1.2.2. Generalized Demodicosis

Generalized demodicosis is when multiple lesions occur on the body or several extremities, or when the entire body area is affected (Figure 3-5). Inflammation and hair loss patches can combine to form vast areas of thickened skin and ulcers. Small alopecia foci are diffuse and scaling, erythema, folliculitis, edema, seborrhea, secondary pyoderma and peripheral lymphadenopathy are seen (1,6). Due to the harm that the parasites do to the hair follicles, secondary bacterial infections are frequently seen in infected dogs, and these lumps may discharge fluid under the skin. Inflammation and hair loss patches can combine to form vast areas of thickened skin and ulcers. Lesions frequently begin around the head, face, and feet, similar to the localized type, but frequently spread to cover broad portions of the body surface. Hyperpigmentation, lymphadenopathy, lethargy, and fever are possible symptoms of severe, generalized pustular demodicosis. Septicaemia related to bacterial infection is conceivable in those seriously afflicted canines and may potentially be deadly (6,11,20).

Figure 3. A case of generalized demodicosis in a 2-year-old Pug

Figure 4. A case of generalized demodicosis in a 1-year-old Anatolian shepherd dog.

Figure 5. A case of generalized demodicosis in a 2-years-old Terrier dog.

1.2.2.1. Pododemodicosis

Although pododemodicosis is mainly seen in the feet, it can cause lesions in different parts of the body (Figure 6,7). The feet are edematous, erythematous, and painful, often with superficial or deep pyoderma (1,21-23).

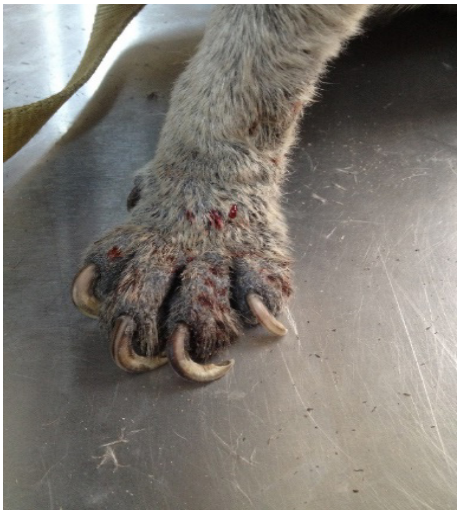

Figure 6. A 2-year-old Pug dog with generalized demodicosis exhibiting pododemodicosis.

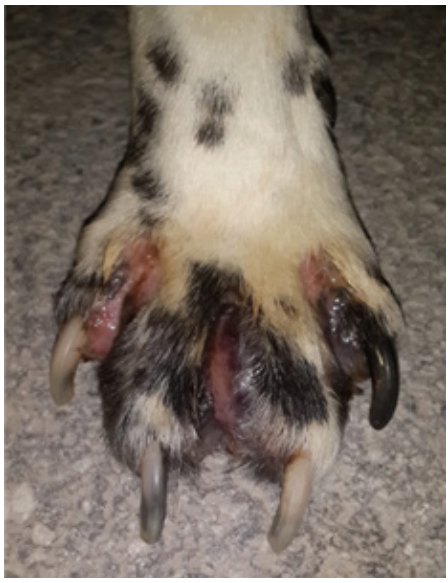

Figure 7. A dog with generalized demodicosis has sores around its feet that are inflamed and painfully discharge.

1.3. Juvenile and Adult Forms of Canine Demodicosis

Demodicosis in dogs is categorized into two different forms, juvenile and adult forms, based on the age of the dogs affected (6).

1.3.1 Juvenil-onset Form

The juvenile form occurs mainly in young dogs and resolves spontaneously within 6-8 weeks. The illness is often generalized from the beginning in 10% of instances. The onset age ranges normally from 3 to 18 months. On the body, erythema, scaling, crusting, alopecia, darker areas of skin, and secondary pyoderma may appear (typically linked to staphylococcal infection, less frequently Pseudomonas and Proteus). There may also be pustules, follicular eruptions, comedones, ulceration, and hemorrhagic bullae. Ordinarily, lesions do not itch unless pyoderma is present. Dogs may develop peripheral lymphadenopathy, anorexia, fever, lethargy, dehydration, and electrolyte imbalances in addition to depression, which is common in them (6,7,11).

1.3.2. Adult-onset Form

The adult form is usually seen in dogs over the age of four. Canines older than four years old with no past history of demodicosis typically develop this

unusual variant. It is usually associated with internal diseases such as Cushing's, neoplasia or immunosuppressive drug therapy. It may also occasionally occur in dogs with atopic dermatitis with a long history of steroid therapy (6,7,11).

1.4. Diagnosis

1.4.1. Skin Scraping

The best diagnostic procedure for identifying demodicosis is thought to be deep skin scrapings. The afflicted skin area should be repeatedly scraped in the direction of hair growth. Squeezing the skin while scraping can help push the mites out from deep within the hair follicles (7,11).

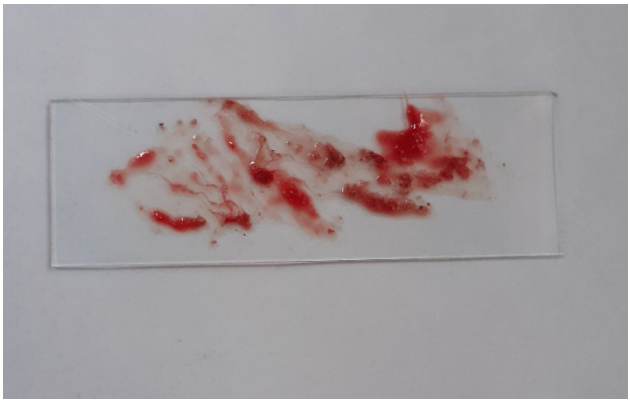

Figure 9. Debris gathered with a deep skin scraping in a dog with generalized demodicosis

1.4.2. Trichogram

The trichogram is an alternative to skin scraping for periocular and interdigital skin areas that are difficult to reach with skin scraping. A sufficient number of hairs (at least 50-100 hairs) should be pulled with forceps and carefully examined under a microscope by dripping mineral or paraffin oil on a slide. Trichograms have high diagnostic value when done correctly (24).

1.4.3. Adhesive Tape/strip

Adhesive strips are an excellent diagnostic tool for canine demodicosis. The adhesive strip is pressed onto the compressed skin with the adhesive side down. The adhesive strip is applied with the adhesive side on a slide and examined under a microscope for mites (25).

1.4.4. Skin Biopsy (histopathological examination)

Skin biopsies performed with histopathological examination, deep skin scrapings, trichograms or adhesive strips can lead to definite results in case of negative results in some body parts (25).

1.4.5. Diagnosis of Bacterial İnfections

There is a strong correlation between secondary bacterial infections and generalized demodicosis (23). A bacterial septicemia is seen, especially in severe cases involving frunculosis (11). Although Gram-negative rods like *Escherichia coli* or *Pseudomonas aeruginosa* may predominate in certain individuals, especially those with furunculosis, *Staphylococcus pseudintermedius* will most frequently be identified. Bacterial culture and antibiogram may be required for these cases (26).

1.5. Treatment

Recommendations for treatment change depending on the type of sickness that is present (27). In most cases, mild localized demodicosis resolves spontaneously without treatment. Some authors suggest that localized demodicosis does not require treatment (13,26). This practice has the potential to mask the risk of developing resistant Demodex strains and potential cases of generalized demodicosis that should be excluded from breeding programs (28). Some authors suggest that cases of localized demodicosis should be treated only topically using lime sulfur or benzoyl peroxide preparations. They recommend treating localized lesions as generalized demodicosis if the lesions multiply or increase in size (20).

In the treatment of demodicosis, if possible, the use of local or systemic corticosteroids, progestogens, or immunosuppressive agents should be avoided (6). These agents inhibit the host immune system's ability to heal and either cause relapses or prevent them. In any case, relapse can happen at stressful times like estrus, pregnancy, lactation, and systemic disorders. If the underlying disease process (spontaneous hyperadrenocorticism) is not treated or diagnosed, adult-onset patients are more likely to occur again. It is important to fully understand the medications used to prevent any potential side effects. This is because there may be certain medications that should not be used concurrently with acaricidal agents (27).

The treatment of generalized and complicated demodicosis with secondary infections can be challenging and time-consuming. One of the most common reasons for treatment failure is premature discontinuation of therapy. Until a second negative skin scraping result is obtained, generalized demodicosis treatment should be observed monthly both clinically and microscopically. Four weeks following the second negative monthly skin scraping result, miticidal therapy should be continued to lower the chance of recurrence. If there is no decrease in mite numbers after a treatment period, especially if active mite reproduction is occurring (presence of eggs, larvae, and nymphs), alternative treatment should be considered (11).

In general, dogs with demodicosis do not require systemic antibiotics, and unless there is a severe bacterial infection present, topical antibacterial treatment combined with effective miticidal agents is usually sufficient (11,12).

There is convincing proof that weekly amitraz baths and macrocyclic lactones such milbemycin oxime, ivermectin, and moxidectin are useful for treating canine demodicosis (29-33). There is a significant incidence of blood brain barrier permeability to ivermectin in some breeds and their crosses, including the Collie, Shetland Sheepdog, Old English Sheepdog, Border Collie, Bearded Collie, and Australian Shepherd (28). To identify dogs that may be sensitive to macrocyclic lactones such as moxidectin and ivermectin, a gradual dose escalation is initially recommended to prevent cases of poisoning caused by these medications (34). Doramectin and eprinomectin are another group of macrocyclic lactone drugs used in the treatment of generalized demodicosis (31,35-38). Imidacloprid and moxidectin are commonly used active ingredients in drug combinations (39,40). Additionally, good results have been reported in the treatment of demodicosis using herbal and homeopathic remedies (41,42).

Until recently, the number of approved medical products for the treatment of demodicosis in dogs has been quite limited. However, the treatment options have significantly expanded with the introduction of a new class called isoxazolines (43,44,45). Recent studies have reported impressive results in the treatment of demodicosis in dogs using isoxazoline group antiparasitic drugs (46-49). Axoksolaner, fluralaner, lotilaner, and sarolaner are active ingredients belonging to the isoxazoline group, which are available in the market. There are numerous oral and topical spot-on products containing these isoxazoline compounds. These isoxazoline group antiparasitic drugs are marketed under various trade names such as BravectoTM (fluralaner, Merck), Bravecto topical

solutionTM (fluralaner, Merck), Bravecto oneTM (fluralaner, Merck), Credelio® (lotilaner, Elanco), Credelio plus® (lotilaner and milbemycin oxime, Elanco), NexGard® (afoxolaner, Boehringer Ingelheim), NexGard spectra® (afoxolaner/milbemycin oxime, Boehringer Ingelheim), Simparica® (sarolaner, Zoetis), and Simparica trio® (sarolaner, moxidectin, and pyrantel, Zoetis). These commercial products consist of either a single isoxazoline compound or a combination of one isoxazoline with 1 or 2 other active ingredients. The licensing of these drugs may vary geographically. Table 1 provides the isoxazoline group antiparasitic drugs and their dosages used in the treatment of demodicosis in dogs. The use of isoxazoline group drugs has become a popular choice in recent years for the treatment of demodicosis due to their ease of use, lower side effects compared to other drugs, and their effectiveness in stubborn cases that do not respond to traditional treatments (43-45,50).

Table 1. Isoxazoline group antiparasitic drugs used in the treatment of demodicosis in dogs all over the world

Product Name (Active ingredient)	Company	Dosage and Administration	How Supplied	Label Use	Extra-label Use
Bravecto™ (Fluralaner) Chews	Intervet/ Merck Animal Health	Orally as a single dose every 12 weeks at the minimum dosage of 25 mg/kg body weight.	Bravecto™ is available in five strengths (112.5, 250, 500, 1000, and 1400 mg fluralaner per chew).	Tick, Flea	Sarcoptic mange, Demodicosis, Lice infestation
Bravecto™ (fluralaner topical solution) for Dogs	Intervet/ Merck Animal Health	Topically as a single dose every 12 weeks at the minimum dose of 25 mg/kg body weight.	Bravecto™ is available in five strengths for use in dogs (112.5, 250, 500, 1000, and 1400 mg fluralaner per tube).	Tick, Flea	Sarcoptic mange, Demodicosis, Lice infestation
Bravecto™ 1-Month (fluralaner) Chews for Dogs)	Intervet/ Merck Animal Health	Orally as a single dose monthly at the minimum dose of 10 mg/ kg body weight	Bravecto™ 1-Month is available in five strengths (45, 100, 200, 400, and 560 mg fluralaner per chew).	Tick, Flea	Sarcoptic mange, Demodicosis, Lice infestation
Simparica® (Sarolaner), Chewables	Zoetis	Orally once a month at the recommended minimum dosage of 2 mg/kg body weight.	Simparica® (sarolaner) Chewables are available in six flavored tablet sizes: 5, 10, 20, 40, 80, and 120 mg.	Tick, Flea	Sarcoptic mange, Demodicosis, Lice infestation
Simparica TRIO® (Sarolaner, moxidectin and pyrantel) Chewable Tablet	Zoetis	Orally, once a month Sarolaner (1.2 mg/kg) moxidectin (24 µg/kg) and pyrantel (5 mg/kg)	Simparica TRIO® (sarolaner, moxidectin, and pyrantel chewable tablets) is available in six flavored tablet sizes (3,6,12,24,48,72 mg)	Heartworm, Roundworm, Hookworm, Tick, Flea	Demodicosis, Sarcoptic mange, Lice infestation

Table 1 (Continued). Isoxazoline group antiparasitic drugs used in the treatment of demodicosis in dogs all over the world

Product Name (Active ingredient)	Company	Dosage and Administration	How Supplied	Label Use	Extra-label Use
NexGard® (Afoxolaner) Chewable Tablet	Boehringer Ingelheim	Orally, once a month, at the minimum dosage of 2.5 mg/kg body weight.	NexGard® is available in four sizes of beef-flavored soft chewables: 11.3, 28.3, 68 or 136 mg afoxolaner.	Tick, Flea, Demodicosis	Sarcoptic mange
NexGard Spectra®, Chewable Tablet (Afoxolaner and milbemycin oxime) Chewable Tablet	Boehringer Ingelheim	Orally every 30 days, at the recommended dosage of 2.5 - 5 mg/kg of afoxolaner and 0.5 - 1 mg/kg of milbemycin oxime.	NexGard Spectra® chewable tablets are available in five soft, beef-flavoured chewable tablets strengths packaged in blister packs of Afoxolaner/Milbemycin Oxime per chewable tablet	Tick, Flea, Demodicosis, Hookworm, Roundworm, Whipworm, Heartworm	Sarcoptic mange
Credelio® (Lotilaner) Chewable Tablet	Elanco	Orally, once a month, at the minimum dosage of 20 mg/kg body weight.	Credelio® is available in five chewable tablet sizes for use in dogs: 56.25, 112.5, 225, 450, and 900 mg lotilaner.	Tick, Flea	Demodicosis, Sarcoptic mange
Credelio Plus® (Lotilaner and Milbemycin oxime) Chewable Tablet	Elanco	Orally, once a month, at the minimum dosage of 20 - 41 mg lotilaner/kg body weight and 0.75 - 1.53 mg milbemycin oxime/kg body weight.	Credelio Plus® is available in five sizes: 56.25/2.11, 112.5/4.22, 225/8.44, 450/16.88, or 900/33.75 mg lotilaner/milbemycin oxime per tablet.	Tick, Flea, Roundworm, Heartworm	Demodicosis, Sarcoptic mange

1.5.1. Adjunctive Therapy

Before starting the treatment, a detailed medical history and clinical examination should be conducted. Owners of dogs with juvenile demodicosis should be asked about potential stress factors, inadequate nutrition, and parasite prevention status. The effectiveness of demodicosis therapy can be improved by lowering stress and maintaining enough nutrition. It is important to feed the dog a complete and balanced diet. Additionally, supplementing with fatty acids may have potential benefits (51). In elderly patients, conditions such as hypothyroidism, hyperadrenocorticism, tumors, or chemotherapy should be considered as potential causes. These possibilities can strain the immune system and trigger mite growth, leading to the progression of the disease. It is important to address underlying illnesses such endoparasitic infections and concomitant disorders (6,11).

A crucial factor for successful demodicosis treatment is owner compliance, so it is important to provide information to the pet owners about the difference between clinical and parasitological improvement. Pet owners should be informed to continue the treatment even after clinical signs have diminished (6,11).

Before starting the treatment, dogs with long hair should be clipped to enhance the direct dermal absorption of the active ingredients. In some cases, removal of crusts may also facilitate the treatment process. However, since this procedure can be painful for dogs, it may require mild anesthesia (27).

A common complication of generalised demodicosis is a secondary bacterial infection of skin (superficial or deep pyoderma, folliculitis, furunculosis) (6,27). Some canines may develop systemic symptoms from pyoderma, including fever, lethargy, anorexia, and an increased likelihood of sepsis and mortality (52). Secondary skin infections must be treated since they frequently cause itching and might bring further comfort to the patient (27,53). *Staphylococcus pseudintermedius* is the most common etiologic agent in dogs, but *S. aureus*, *S. schleiferi*, and less commonly Gram-negative bacteria such as Pseudomonas or Proteus may also be involved (7,23). Topical antimicrobials and systemic antibiotics are often used to treat bacterial pyoderma (7). Affected body areas or the entire animal can be washed in cases of secondary pyoderma, seborrhea, or when the skin is dirty. Shampoos containing lime sulfur, benzoyl peroxide, chlorhexidine, or ethyl lactate are suitable for this purpose. This application can be done on a weekly basis or more frequently as needed (6,51). If the use of antibiotics is necessary, they should be administered after bacterial culture

and antibiotic susceptibility testing. (8,54) Complicated cases of demodicosis associated with pyoderma may require 4-6 weeks of antibiotic treatment with agents such as amoxicillin + clavulanic acid, bacitracin, cefadroxil, cefovecin sodium, cefpodoxime proxetil, fluoroquinolones, and cephalexin (7,23,55).

2. Conclusion

An effective cure for generalized demodicosis is becoming increasingly realistic thanks to new therapy choices. For the treatment of canine demodicosis, amitraz, macrocyclic lactones, and more recently, isoxazolines, have all shown to be effective (11,12). It can be difficult to treat canine demodicosis since there are so many variables to take into account, such as the kind of demodicosis (localized or widespread), the patient's history and breed, underlying reasons, and client preferences (20). Early identification and treatment of this condition will benefit from the technician's involvement in gathering a complete history, doing correct skin scrapings, and giving effective client education about this disease. Treating secondary infections or underlying diseases can significantly improve the patient's comfort and prognosis, even if achieving a parasitological cure using currently available therapies may take a while (45,50).

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CHAPTER III

ANTIPARASITIC DRUGS USED THE IN TREATMENT OF DEMODICOSIS IN DOGS: 2022 LITERATURE REVIEW

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1. Introduction

There is strong evidence that canine demodicosis can be effectively treated with weekly amitraz baths and macrocyclic lactones such milbemycin oxime, ivermectin, and moxidectin. Another class of macrocyclic lactone medications used to treat generalized demodicosis is doramactin. Active components in medication combinations frequently include imidacloprid and moxidectin. There have only lately been a few authorized medicinal products for the treatment of canine demodicosis. Until recently, the number of approved medical products for the treatment of demodicosis in dogs has been quite limited. With the use of antiparasitic medications from the isoxazoline group, recent studies have demonstrated remarkable outcomes in the treatment of canine demodicosis. Afoxolaner, fluralaner, lotilaner, and sarolaner are active ingredients belonging to the isoxazoline group, which are available in the market. There are numerous oral and topical spot-on products containing these isoxazoline compounds. The use of isoxazoline group drugs has become a popular choice in recent years for the treatment of demodicosis due to their ease of use, lower side effects compared to other drugs, and their effectiveness in stubborn cases that do not respond to traditional treatments (1-6).

2. Amidines

2.1. Amitraz

Amitraz is an externally applied antiparasitic drug with acaricidal and insecticidal effects. It is a monoamine oxidase and prostaglandin synthesis inhibitor and functions as an α-2 adrenergic receptor agonist (1,2). Several drugs containing the active ingredient amitraz have been approved in our country. These include KENAZ 12.5% EC (Atabay Kimya Sanayi AŞ/ Kocaeli, Turkey, License Date: 1981), AMITRAX 12.5% (amitraz 125 mg/1ml, Teknovet, Istanbul, Türkiye, License Date: 2008), OVATOKS (amitraz 125 mg/1ml, İlteriş, Istanbul, Türkiye, License Date: 2003), and Apivar® (Veto Pharma SAS, 500 mg impregnated strip, France). These products, primarily licensed for use in cattle, sheep, and bees, are commonly used off-label for the treatment of ectoparasitic diseases in dogs and other pets (such as tick infestations, lice, mange, demodicosis, etc.) (3-5). Demodicosis in dogs has been successfully treated with weekly amitraz baths at dosages of 0.025 to 0.05. To ensure proper penetration of the medication into the skin, it is recommended to trim the fur of long-haired animals (6).

However, poisoning cases in dogs are frequently reported due to the improper use of amitraz. Since amitraz is a monoamine oxidase inhibitor, concurrent use of serotonergic drugs (such as hydroxyzine, tricyclic antidepressants, and antihypertensives) in animals or their owners can lead to adverse effects. After the application of amitraz, if adequate ventilation of the environment is not ensured, various side effects have been reported in both patients and owners. Undesirable side effects such as headache, asthma, sedation, bradycardia, polyuria, hypothermia, hyperglycemia, itching, polydipsia, anorexia, ataxia, seizures, and even death have been described. According to Aytekin et al. (7), a dog presented to the clinic with complaints of vomiting, tremors, hypersalivation, and anorexia, which were attributed to amitraz toxicity. The physical examination of the dog revealed tonic and clonic seizures, depression, pallor of the conjunctival mucosa, bradycardia, hyperthermia, and increased respiratory rate. It should be noted that puppies under the age of 12 weeks and Chihuahua breed dogs are known to be sensitive to amitraz. Additionally, there is limited research available regarding the use of amitraz in pregnant animals. Therefore, alternative treatment options should be considered for these types of animals (8-11).

The mite count fell by 42% and 94% at 14- and 28-days following therapy, respectively, in a case study examining the efficiency of metaflumizone + amitraz spot formulation (ProMeris Duo®; Fort Dodge) against generalized demodectic mange. Additionally, it was noted that no nymphs or larvae were detected from day 28 onwards after treatment (12).

Another study reported by Arslan and Açıcı (3) described a case of localized demodicosis in a nine-month-old German Shepherd dog, which was successfully treated with a combination therapy of benzoyl peroxide and amitraz within a very short period of two weeks. It was reported that the dog did not experience any recurrence of localized or generalized demodicosis during a two-year follow-up.

Amitraz (Mitaban®, 0.025% solution, Pfizer) has been used for years in the United States for the treatment of demodicosis in dogs and is an FDA-approved compound for this purpose (13,14). In a field study conducted by Folz et al. (15) to evaluate the effectiveness and safety of Mitaban® for the treatment of demodicosis in dogs, it was found that 99.2% of 252 dogs with localized demodicosis were clinically normal and no mites were detected in skin scrapings after 3-18 days of treatment. Similarly, in the same study, it was diagnosed that 99.1% of 569 animals with generalized demodicosis were free

of mites after 3-18 days of treatment. The most commonly reported side effect in treated animals was mild sedation, which occurred in 9.2% of all patients approximately 6-8 hours after treatment. Additionally, mild itching was reported in 2.4% of dogs. ProMeris Duo® (metaflumizone 150 mg/ml, amitraz 150 mg/ml, Pfizer, New York, USA) is a Spot-on combination product that was approved for use in dogs in the United States and Europe in 2007 (16). It has been used by many researchers for the treatment of localized and generalized demodicosis, with successful results (16,17). However, ProMeris Duo® has been withdrawn from the market upon the request of the marketing authorization holder. In 2007, the effectiveness of ProMeris Duo® was tested on 16 dogs diagnosed with demodicosis. The first group, consisting of eight dogs, was treated with ProMeris Duo® at a dose of 20 mg/kg on days 0, 28, and 56. The second group was treated with the same dose of ProMeris Duo® on days 0, 14, 28, 42, 56, and 70. On day 84, the success rate in both groups was determined to be 42.9% and 62.5%, respectively. Mild gastrointestinal symptoms were reported as side effects, but these side effects were not attributed to the administration of the drug (17). In another study conducted in 2011, it was reported that pemphigus foliaceus-like skin reactions were observed in 22 dogs after treatment with ProMeris Duo®. Among these dogs, local changes were observed at the application site in 8 dogs, while distant skin lesions were observed in the remaining 14 dogs. Three dogs with localized skin lesions exhibited systemic symptoms of this autoimmune disease, and it was determined that immunosuppressive treatment was required for these dogs. In the remaining 14 cases, systemic symptoms were identified in 11 dogs, and it was concluded that 10 of these dogs required treatment with immunosuppressive therapy (18).

Certifect® (fipronil 6.7 mg/kg, amitraz 8.0 mg/kg, (S)-methoprene 6.0 mg/kg body weight; Merial, Lyon, France), containing amitraz, has been approved and marketed for dogs in Europe. It was introduced to the market in 2011. It has been used by many researchers for the treatment of localized and generalized demodicosis, and successful results have been achieved (19,20). However, the marketing authorization for Certifect® has been withdrawn from the market at the request of the license holder.

In a 2013 research, 18 dogs with generalized demodicosis were studied to see how Certifect® affected them. No side effects were observed during the treatment, and it was reported that one dog died during the study due to the complex course of demodicosis and subsequent septicemia with secondary bacterial infection (21). However, in a study conducted in 2014, side effects

were observed in 21 dogs treated with Certifect®, manifesting as skin reactions resembling the clinical presentation of pemphigus foliaceus. These changes occurred locally at the application site in 6 dogs and as distant skin reactions in 15 dogs. Additionally, systemic symptoms were reported in 9 animals. The study concluded that Certifect® may induce an acantholytic pustular dermatitis that resembles pemphigus foliaceus clinically and histologically (22).

Pekmezci et al. (4) conducted a study where they compared the efficacy of a combination treatment of amitraz (KENAZ %12.5 EC, Atabay) + inactivated papoxvirus ovis (Zylexis, Zoetis) with a group treated solely with amitraz (KENAZ %12.5 EC) in cases of generalized demodicosis. The study reported that the group treated with the combination therapy showed better clinical improvement and total clinical scores compared to the group treated with amitraz alone. Furthermore, neither treatment group in which amitraz was used for therapy experienced any adverse reactions to the medication. The efficacy of oral ivermectin, topical amitraz, and their combination in the treatment of canine generalized demodicosis was examined in a 2019 research. The study included 12 dogs, with four animals in each group. Over the course of 45 days, various treatment regimens were used. After the 45-day treatment period, it was reported that dogs treated with combination therapy showed faster improvement compared to those treated with either oral ivermectin or topical amitraz alone (23).

A 10-month-old German Shepherd dog diagnosed with generalized demodicosis was treated with a 0.2% amitraz solution, administered as baths three times a week and spray application on other days. After 45 days of treatment, the case of generalized demodicosis showed complete recovery, and no recurrence of demodectic mange was observed during the subsequent six-month follow-up period (5).

In cases of generalized demodicosis in dogs, Parwari et al. (24) examined the effectiveness of amitraz, doramectin, imidacloprid + moxidectin, a test product (Green clean), and their combinations. For the treatment experiment, a total of 48 patients of generalized demodicosis were chosen. The affected dogs were divided into six groups (Groups A, B, C, D, E, and F), with 8 dogs in each group. A control group (Group G) of six healthy canines free of Demodex mites was employed for comparison. The therapeutic trial's enrolled patients were examined clinically and parasitologically at weekly intervals until either clinical improvement (4–7 weeks) or treatment failure was determined. The treatment regimens consisting of systemic miticidal drugs (doramectin, 600 µg/kg SC,

weekly) and a topical miticidal compound (amitraz, 500 ppm spray, once a week or twice daily with the test product, 5% spray) along with supportive therapy were reported to be more effective in resolving lesions and eliminating mites compared to single-drug (doramectin/amitraz/test product) treatment regimens. Significant therapeutic results were obtained with combination therapy and supportive treatment using benzoyl peroxide shampoo (24).

In an another study, 18 out of 23 dogs with positive canine demodicosis included and 6 healthy dogs as a control group. There were 6 dogs in each of the 4 treatment groups, which were split among the dogs: Group 1: Control, Group 2: Amitraz 12.5% (0.05%, topical, once a week for 4 weeks) + Ivermectin (0.2 mg/kg, once a week for 4 weeks, subcutaneous), Group 3: Saffron oil (topical, for 15 days) + Saffron tablet (for 10 days, orally), Group 4: Fluralaner tablet (25 mg/kg, single dose, orally). In Group 2, it was reported that the number of mites decreased by 29.7% and 100% on the 15th and 30th days of treatment, respectively, along with concurrent clinical improvement. This study reported that all treatment protocols were effective, but the combination of ivermectin and amitraz was found to be more effective than other treatment protocols (25).

In a study, a total of 60 cases of Demodex mange, co-infected with tick or flea infestation, four groups, which were formed (Group A: Control, Group B: Amitraz, Group C: Deltamethrin, Group D: Ivermectin), with 15 dogs in each group. Dogs in Group B were topically treated with recommended doses (2 ml/ litre) of 0.2% amitraz solution [(AMT) 5% w/v, 60 ml bottle] for consecutive 4 weeks at weekly intervals. The recovery rate of dogs treated with topical amitraz was determined to be 60%. Additionally, it was reported that the amitraz treatment protocol had a higher recovery rate versus the control group and other treatment protocols (ivermectin, deltamethrin) (26).

Yarim et al. (27) carried out research on dogs with generalized demodicosis, examining the role of inactive parapoxvirus (IPPVO) in addition to amitraz treatment in maintaining skin integrity and promoting wound healing, along with clinical improvement. In the study it was found that the administration of IPPVO led to a significant decrease in the circulating concentrations and dermal expressions of insulin-like growth factor (IGF)-1 and -2, epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF). These findings suggest that IPPVO may have an impact in modulating these growth factors, contributing to the healing process and preservation of skin integrity in dogs with generalized demodicosis.

3. Macrocyclic Lactones

3.1. Ivermectin

Avermectins, including ivermectin, are characterized by a 16-membered lactone ring structure, which is derived from the fermentation product of *Streptomyces avermitilis* (28). The lactone ring structure of avermectins contains a hexahydrobenzofuran, a disaccharide group at C-13, and a spiroketal ring between C-17 and C-28 (29). Ivermectin, also known as 22,23-dihydroavermectin B1, is a modified derivative of naturally occurring avermectin B1. It consists of approximately 80% 22,23-dihydroavermectin B1a and 20% 22,23-dihydroavermectin B1b (30,31). Ivermectin is widely used as a broad-spectrum antiparasitic agent in various internal and external parasites in cattle, sheep, goats, dogs, cats, horses, and pigs, administered by injection, topical, or oral application in doses ranging from 200 to 500 μg/kg (32-37). Neurotoxicity induced by ivermectin has been demonstrated in various studies in certain dog breeds (38,39). These dog breeds include primarily Collies (54.6%), Shetland Sheepdogs (30%), Australian Shepherds (19.5%), Whippets (18.5%), English Shepherds (6.3%), and Border Collies (0.6%) (40,41). The reason for neurotoxicity is a mutation in the ABCB1 gene, which leads to insufficient or absent synthesis of P-glycoprotein (P-Gp). This protein plays an important role in the physiological barriers, such as the blood-brain barrier, blood-testis barrier, and placental barrier, which restrict the entry of drugs (6). Accumulation of ivermectin in the central nervous system can lead to neurotoxic effects characterized by ataxia, lethargy, coma, tremors, mydriasis (dilated pupils), and vision loss (42).

In a study conducted in 1996, the efficacy of ivermectin was evaluated in twelve dogs diagnosed with juvenile or adult-onset demodicosis. The dogs included in the study were treated with a diluted ivermectin solution (0.4 mg/kg oral, daily) licensed for use in horses (Eqvalan®, Oral Pat, MSD, USA) or cattle (Ivomec®, MSD, USA). Among these dogs, five achieved recoveries after an average of 15 weeks of treatment. Two out of the remaining seven dogs showed positive skin scrapings during follow-up examinations, while in five dogs, sufficient treatment efficacy with ivermectin could not be achieved due to concurrent other diseases. Among the five dogs with concurrent other diseases, one had colitis, one had hyperadrenocorticism, two had hypothyroidism, and one had pemphigus foliaceus, all of which were diagnosed and treated prior to the study. The dog with colitis had previously been treated with sulfasalazine

(Azulfidine®, NY, USA), and the dog with pemphigus foliaceus had been treated with methylprednisolone and aurothioglucose (Solganol®, NJ, USA). A St. Bernard dog with a pre-existing condition of hypothyroidism developed symptoms of muscle weakness and tremors suggestive of intoxication after five weeks of treatment. Three days following the end of the medication, these negative effects disappeared (43).

In a study conducted by Beyazıt et al. (33), an 8-year-old German Shepherd dog diagnosed with pododemodicosis was treated with a combination of ivermectin, an antibacterial medication, and beta-glucan. At the end of the first month, a reduction in the number of Demodex spp. was observed, and by the end of the second month, both clinical and microscopic improvements were noted. Furthermore, during a follow-up examination six months later, complete healing of the lesions was reported.

Maden et al. (44) reported in a study in which a 5-year-old English Pointer dog with atypical dermatitis caused by *Demodex canis* was successfully treated with ivermectin (0.6 mg/kg) administered at 3-day intervals for a duration of 4 months. The treatment was reported to be effective and did not result in any complications.

Fernandez et al. (45) conducted a study to investigate the effectiveness of homeopathic remedies in the treatment of demodicosis in dogs. The study divided the dogs into two treatment groups at random. In Group 1 (n=30), the dogs were treated with a combination of ivermectin (0.1 ml/5 kg, twice weekly, for 4 weeks), the antibiotic Gentamycin (6-10 mg/kg, once daily, for 10 days), Yatren (casein containing yatrenic acid/protoalbumin, 0.5-5 ml, once weekly, for one month), and topical amitraz (1 ml per liter of water, twice weekly, for one month). In Group 2 (n=30), the dogs were treated with the same treatment protocol as Group 1 along with Sulphur 30 CH (five sublingual drops, every 12 hours, for one month). It was reported that the use of homeopathic remedies (Sulphur 30 CH) in conjunction with conventional medications was effective in achieving recovery without relapse in animals affected by *Demodex canis*.

In a study evaluating the chemotherapeutic management in 96 dogs with demodicosis of different ages and genders, the dogs included in the study were treated with benzoyl peroxide shampoo, ivermectin, amitraz, antibiotic (Cephalexin-600), and multivitamin syrup. Skin scraping samples taken on days 21 and 28 after treatment showed no presence of mature or developing stages of mites, and it was reported that all dogs had completely recovered (46).

In a study conducted in 2021, the effectiveness of a combination treatment consisting of ivermectin (600 mcg/kg body weight, SC, once a week), amitraz

(12.5% solution, topical, once a week), oral immunomodulators (Immunol suspension, 3-5 ml, twice a day, for 45 days), and antibiotics (Cephalexin tablet, 25 mg/kg, twice a day, for 45 days) to prevent secondary pyoderma was investigated. It was reported that significant clinical improvement was observed at the end of the 45th day of treatment (47).

Pem et al. (26) investigated a total of 60 cases of Demodex mange, co-infected with ticks or fleas. The infected dogs were divided into four groups (Group A: Control, Group B: Amitraz, Group C: Deltamethrin, Group C: Ivermectin) with 15 dogs in each group and treated with different medications. Dogs in Group C were treated with ivermectin at the recommended doses (0.1 mg/kg, SC, once a week) for four consecutive weeks. The recovery rate of dogs treated with ivermectin was determined to be 53.3%. However, it was reported that the recovery rate was lower with the amitraz (Group B) treatment protocol (26).

Atif et al. (36) reported a study, evaluating the therapeutic efficacy of ivermectin in an elderly Labrador Retriever dog diagnosed with demodicosis (*Demodex canis*). The treatment protocol consisted of oral ivermectin tablets (0.6 mg/kg/day) for two weeks, antimicrobial shampoo (every three days, for two weeks), and supportive care. By the third week of treatment, complete recovery was observed in the dog, and no complications were reported. Furthermore, Demodicozis (*Demodex canis*) diagnosed in 19 dogs (11 generalized, 8 localized forms) was reported to have improved within 4 weeks with a combination of ivermectin (0.3 mg/kg, SC, once a week, for 4 weeks), cefadroxil (5 mg/kg, once daily, for 7 days), topical povidone iodine solution (10% w/v), benzoyl peroxide shampoo, and supportive treatment (48).

In a study reported by Rathod et al. (49), a 3-year-old Pug dog diagnosed with demodicosis was treated with ivermectin (0.2 mg/kg, SC, once a week) for 8 weeks along with supportive therapy. Additionally, bathing with benzoyl peroxide shampoo followed by topical application of amitraz (2 ml in 1 liter of water) twice a week was recommended until complete recovery. Two months after the treatment, it was reported that the dog's overall skin lesions had improved, there were no demodex mites in the skin scrape samples, and the hair had regrown.

3.2. Doramectin

Doramectin (Dectomax, Pfizer Animal Health) is a long-acting avermectin used in dogs at a dose of 600 µg/kg (subcutaneous) once a week. (50,51) Doramectin, also known as 25-cyclohexyl-5-O-demethyl-25-de(1-methylpropyl)-Avermectin A1a, is characterized by a cyclohexyl ring at C25.

It is closely related to the active ingredient ivermectin. It is produced through mutational biosynthesis from a strain of Streptomyces avermitilis and exhibits similar activity to avermectin B components (52). This drug should not be used in ivermectin-sensitive breeds (i.e., collie, Shetland sheepdog, Australian shepherd, Old English sheepdog, and other herding breeds) (53).

In a retrospective study conducted in the United States, the efficacy and safety of weekly subcutaneous injections of doramectin (Dectomax®, Pfizer Animal Health, USA) at a dose of 0.6 mg/kg body weight were investigated in 232 dogs diagnosed with generalized demodicosis. Starting from the fourth injection, all dogs were examined for demodex mites through consecutive skin scrapings, and if two consecutive skin scraping results were negative, the treatment was discontinued. The duration of treatment for patients ranged from 4 to 20 weeks, with an average treatment duration of 7.1 weeks. Three dogs required retreatment after one month, while the remaining ten dogs did not show a reduction in mite count after treatment. This study reported remission in 94.8% of dogs treated with weekly subcutaneous doramectin injections at a dose rate of 0.6 mg/kg body weight. Two dogs, a 7-year-old female Lhasa Apso with neurological symptoms and an 8-month-old male dog with a local reaction to the injection, had dropped treatment due to side effects (54).

In a study conducted in 2018, the efficacy of subcutaneously injected doramectin compared to orally administered medication was evaluated in 29 dogs diagnosed with generalized demodicosis. The first group, consisting of 16 dogs, received doramectin via subcutaneous injections at a dose of 600 µg/kg body weight once a week. The second group, consisting of 13 dogs, received oral doramectin at a dose of 600 µg/kg body weight twice a week. The first negative skin scraping time was reported as 7.2 weeks in the first group and 7.4 weeks in the second group. The success rates were 81% and 92% in the first and second groups, respectively. The treatment success rate was reported to be the same for both protocols. Within the following twelve months, three dogs from the first group and one dog from the second group experienced a relapse. No adverse effects were reported in any of the included patients in the study (55).

Praveen et al. (56), evaluated the effectiveness of doramectin in combination with ivermectin in a case of complicated generalized demodicosis with secondary bacterial infection in a 2-year-old Beagle dog. The dog was treated with oral ivermectin (500 µg/kg) for seven weeks, along with weekly parenteral doramectin (SC, 0.2 mg/kg). Topical amitraz (2 ml in 1 liter of water) was applied once a week, followed by bathing with benzoyl peroxide shampoo

until the recovery period. As adjunctive therapy for the treatment of secondary bacterial infection, oral cephalexin tablets (20 mg/kg BID) were administered. It was reported that the dog achieved complete recovery without any side effects after seven weeks of treatment with doramectin, amitraz, and supportive therapy.

3.3. Moxidectin

Moxidectin is a semi-synthetic product derived from the fermentation of Streptomyces species. It is a macrocyclic lactone compound used for the treatment of generalized demodicosis in dogs. (57) Advocate® (containing 2.5 mg/kg Moxidectin and 10 mg/kg Imidacloprid, Bayer Animal Health GmbH, Germany) is a commercial product available in the market. This medication is effective against both endoparasites and ectoparasites in dogs and cats. Its efficacy and safety have been tested by multiple researchers (58-62). Mild side effects such as vomiting, salivation, decreased appetite, lethargy, difficulty in breathing, and facial swelling have been reported in dogs following the administration of Moxidectin (63,64). Moxidectin can be administered both orally and subcutaneously. However, it is recommended to administer it orally due to a higher incidence of the mentioned side effects with subcutaneous administration (63). Neurological symptoms such as mydriasis, tremors, ataxia, and seizures have been reported in cases of overdose or toxicity similar to ivermectin (42,65,66). The combination of imidacloprid (10%) + moxidectin (2.5%) has been well tolerated even at five times the recommended dose in breeds sensitive to ivermectin, with a monthly application of three doses (62). In a study comparing different application rates of moxidectin/imidacloprid spot-on, a significant dose-dependent effect was observed, and it was reported that more frequent application, such as monthly or more frequently, increased the effectiveness of the medication (67).

A study conducted at the Veterinary Faculty of the University of Vienna in 2000 evaluated the efficacy of an approved formulation for cattle on 63 dogs and seven rabbits against Sarcoptes scabiei, Demodex spp., and Psoroptes cuniculi mites. Among the dogs included in the study, 22 were diagnosed with generalized demodicosis. Oral moxidectin (Cydectin® 1% solution for injection for cattle, Zoetis) was then given to the dogs at a dose of 0.1 mg/kg body weight. This dose was increased by 0.1 mg/kg body weight every day and adjusted to 0.4 mg/kg body weight from the fourth day if no side effects were observed. The treatment was continued for 42-120 days, and successful results were achieved in 16 dogs, with two negative skin scrapings and no relapse observed during

the subsequent 12 months. The study was prematurely terminated because three animals did not return to the clinic. Among the remaining three dogs, ataxia, lethargy, vomiting, and anorexia were reported as adverse effects, leading to their exclusion from the study (63).

Arsenovic et al. (68) evaluated the efficacy of conventional treatments for demodicosis in dogs by analyzing published articles from 1980 to 2014 using mathematical analysis. They examined 106 clinical studies that recorded 3414 cases of generalized demodicosis in dogs. The results of these studies reported that moxidectin spot-on (Advocate®, Bayer, administered weekly) showed better results compared to other medications such as amitraz, doramectin, and ivermectin. In a study conducted in 2016, sixteen dogs with generalized demodicosis were treated with NexGard® (8 dogs, afoxolaner, Böhringer Ingelheim Vetmedica GmbH and Advocate® Spot-on (eight dogs, 10 mg/kg Imidacloprid, 2.5 mg/kg Moxidectin, Bayer Animal Health GmbH). NexGard® was administered orally at a minimum recommended dose of 2.5 mg/kg body weight on days 0, 14, 28, and 56. Dogs treated with NexGard® showed a reduction of 99.2%, 99.9%, and 100% in mite numbers on days 28, 56, and 84, respectively. Dogs treated with Advocate® showed a reduction of 89.8%, 85.2%, and 86.6% in mite numbers on days 28, 56, and 84, respectively. Therefore, it was reported that Advocate® had lower efficacy compared to NexGard® in the treatment of generalized demodicosis (69).

In a laboratory study conducted in 2019, the efficacy of two topical spot-on treatments, fluralaner (Bravecto™ Spot-on Solution, Merck Animal Health, Madison, NJ, USA) and the combination of imidacloprid and moxidectin (Advocate®, Bayer Animal Health, Leverkusen, Germany), was compared in naturally infected dogs with generalized demodicosis. Sixteen dogs, divided randomly into two study groups of eight dogs each, were included in the study. On day 0, dogs in Group 1 were treated once with fluralaner spot-on solution. Dogs in the other group were treated with imidacloprid/moxidectin spot-on solution either three times (on days 0, 28, and 56) or weekly in severe cases. After the application of fluralaner, miticidal efficacy was determined as 99.7% on day 28, >99.9% on day 56, and 100% on day 84. In dogs treated topically with imidacloprid and moxidectin combination, miticidal efficacy was determined as 9.8% on day 28, 45.4% on day 56, and 0% on day 84, which was significantly lower ($P<0.01$) than the group treated with fluralaner at all time periods. A single topical application of fluralaner has successfully eliminated Demodex spp. mites in dogs with generalized demodicosis. However, the topical treatment

with imidacloprid and moxidectin combination, administered at 28-day intervals three times or more frequently, was unable to completely eliminate the mites in the majority of treated dogs (70). Although new drugs (Fluralaner) were shown to be very effective in above study comparing old drugs (imidacloprid/ moxidectin), when the results of the article are examined individually, 2 outlier data can be seen. When these two outlier data are removed, it will be seen that the effectiveness of old drugs are close to the results obtained with Fluralaner.

Indeed, in a case study reported in 2021, successful results were obtained with a combination of moxidectin, amitraz and supportive therapy in a dog diagnosed with generalized demodicosis at 10 months of age. The dog received four oral administrations of moxidectin (0.3 mg/kg, SC) at seven-day intervals and amitraz (250 ppm, bath) four times at weekly intervals. In the dog treated with this combination, it was determined that almost all of the hair had grown back at the end of the 45th day and a positive clinical improvement was achieved. In addition, it has been suggested to support the treatment with shampoo containing benzoyl peroxide, drugs such as immunostimulants and antibiotics (71).

3.4. Milbemycin Oxime

Milbemycin oxime is a semi-synthetic derivative of milbemycin A3/ A4, which is a natural fermentation product of Streptomyces hygroscopicus aureolacrimosus (72,73). Indeed, milbemycin oxime is available in the market under various trade names, including Interceptor® (milbemycin oxime), NexGard Spectra® (milbemycin oxime/ afoxolaner by Merial), and SENTINEL® Tablet (milbemycin oxime/ lufenuron). Milbemycin oxime (Interceptor®) is approved for the prevention of heartworm disease and control of intestinal parasites in dogs older than 4 weeks of age in the United States. It can safely be used in puppies that are four weeks of age and older, weighing 900 grams or more. Successful results have been reported when using milbemycin oxime in the treatment of demodicosis in dogs at a dosage range of 0.5-2.0 mg/kg (oral, every 24 hours) (74). Additionally, it is stated that more effective results are obtained from higher doses of milbemycin oxime (1-2 mg/kg). (75-77) Milbemycin is stated to be better tolerated by dogs compared to other macrocyclic lactones and has a higher safety margin. (78-80) However, it should be used with caution in dog breeds with the ABCB1-1Δ gene mutation (such as Border Collie, Shetland Sheepdog, Australian Shepherd Dog, English Shepherd, etc.) due to reported neurological side effects. (9,42,81) Milbemycin oxime is also effective against various internal parasites including *Dirofilaria immitis*, *Ancylostoma caninum*,

Toxocara canis, *Toxascaris leonina*, *Trichuris vulpis*, and *Spirocerca lupi* (69,82,83).

Several researchers have recommended the successful use of milbemycin oxime in the treatment of generalized demodicosis (9,74-77).

A retrospective study was conducted to evaluate the efficacy of milbemycin oxime (Interceptor®, Ciba-Geigy, Greensboro, USA) in dogs diagnosed with generalized demodicosis. The study was carried out on 99 dogs between 1995 and 2000. It was determined that 53% of the included dogs had juvenile demodicosis, while 47% had adult demodicosis. Following treatment with Interceptor® chewable tablets (0.5-1.6 mg/kg body weight, oral), 85% of the dogs were successfully treated within a period of 1-6 months (average of 2.3 months). No live mites or recurrences were observed in skin scrape samples within the following 12 months. It was found that the recovery rate in young animals (with a mean age of 2.97 years in treated cases) was significantly better compared to older animals (with a mean age of 8.02 years in untreated cases). It was reported that severe pododemodicosis cases had lower recovery rates, with 9 out of 11 dogs remaining untreated (74).

Arsenovic et al. (68) evaluated the efficacy of milbemycin oxime (0.5 mg/kg, daily, oral) and moxidectin spot-on (Advocate®, Bayer, once a week) applications have yielded better results compared to other drugs (such as amitraz, doramectin, ivermectin) in the treatment of demodicosis. However, due to the disadvantages of milbemycin oxime, such as its short half-life (2-3 days) and the need for daily dosing, it seems more reasonable to use it in combination with afoxolaner or other compounds rather than as a standalone treatment for demodicosis (84).

In a study investigating the safety of the afoxolaner/milbemycin oxime combination, it was reported that when administered consecutively at doses up to 5 times, the maximum exposure dose in dogs under 8 weeks of age (afoxolaner/milbemycin, 5mg/kg, oral), it was deemed safe and no adverse effects related to the medication were observed. (80) Thirty-two healthy 8-weeks-old Beagle puppies (16 males and 16 females) were included in the study and randomly assigned to one of four treatment groups based on drug doses (0 mg/kg, 1 mg/kg, 3 mg/kg, 5 mg/kg). Three doses were administered at 28-day intervals (on days 0, 28, and 56), followed by three additional doses at 14-day intervals (on days 84, 98, and 112). Throughout the study, physical examinations, clinical pathology analysis, and blood samples for afoxolaner and milbemycin oxime plasma concentrations were collected. No changes related to afoxolaner/

milbemycin oxime treatment were observed in growth, physical examination findings, or tissues examined histopathologically. There were no signs of macrocyclic lactone sensitivity observed at any time during the study (80).

4. Izoxazolines

"Izoxazolines" are a family of medications that have recently been shown to be successful in treating canine demodicosis (85,86). Recent research has shown that compounds from the izoxazoline family have the potential to be a safe and efficient therapy for generalized demodicosis, particularly in situations when conventional treatments are ineffective (87-90). The izoxazoline group includes compounds such as afloxolaner, fluralaner, sarolaner, and lotilaner. The oral chewable formulations of afloxolaner and fluralaner were introduced to the market in 2013-2014, followed by sarolaner in 2015, and lotilaner in 2017 (85). The ektoparasitic drugs in this group are marketed under commercial names such as NexGard® (afoksolaner, Boehringer Ingelheim), Bravecto® (fluralaner, Merck), Simparica® (sarolaner, Zoetis), and Credelio® (lotilaner, Elanco). Additionally, there are combination products available such as NexGard Spectra® (afoksolaner and milbemycin oxime, Boehringer Ingelheim), Bravecto Plus® (fluralaner and moxidectin, Merck), Simparica Trio® (sarolaner, moxidectin, and pyrantel, Zoetis), and Revolution Plus® (selamectin and sarolaner, Zoetis). The isoxazoline group of drugs available in Türkiye is provided in **Table 1**. These molecules inhibit chloride channels by causing inhibition of γ-aminobutyric acid (GABA) channels, which act as neurotransmitters in the central nervous system. As a result, they block the passage of chloride ions across the pre- and post-synaptic cell membranes. Excessive stimulation caused by isoxazoline leads to uncontrolled activity in the central nervous system, ultimately resulting in the death of insects and mites (91,92). According to our knowledge, no resistance has been reported against isoxazolines (85).

Table 1. Licensed isoxazoline group antiparasitic drugs for dogs in Türkiye.

Product Name (Active ingredient)	Company	Dosage and Administration	Label Use	Extra-label Use
Simparica® (Sarolaner), Chewables	Zoetis	Orally once a month at the recommended minimum dosage of 2 mg/kg body weight.	Tick, Flea	Sarcoptic mange, Demodicosis, Lice infestation
Bravecto™ (Fluralaner) Chews	MSD Animal Health	Orally as a single dose every 12 weeks at the minimum dosage of 25 mg/kg body weight.	Tick, Flea	Sarcoptic mange, Demodicosi, Lice infestation
NexGard® (Afoksolaner) Chewable Tablet	Boehringer Ingelheim	Orally, once a month, at the minimum dosage of 2.5 mg/kg body weight.	Tick, Flea, Demodicosis	Sarcoptic mange
NexGard Spectra®, Çiğnenebilir Tablet (Afoxolaner and milbemycin oxime) Chewable Tablet	Boehringer Ingelheim	Orally, every 30 days, at the dosage of 2.5–5 mg/kg of afoxolaner and 0.5–1 mg/kg of milbemycin oxime.	Tick, Flea, Demodicosis, Hookworm, Roundworm,Whipworm, Heartworm	Sarcoptic mange

4.1. Afoxolaner

The afoxolaner is marketed under the trade names NexGard™ (afloxolaner, Merial, Lyon, France) and NexGard Spectra™ (afoxolaner 9-150 mg/kg, milbemycin oxime 1.8-30 mg/kg, Merial, Lyon, France). Afoxolaner is used for the treatment of ticks, fleas, demodicosis, sarcoptic mange, and Otodectes cynotis in dogs (69,87,93-98).

In a study conducted by Beugnet et al. (87), eight dogs with generalized demodicosis were treated with NexGard® (Boehringer Ingelheim Vetmedica GmbH). On days 0, 14, 28, and 56, the prescribed dosage of NexGard® (at least 2.5 mg/kg body weight) was given orally. It was reported that there was a reduction of 99.2%, 99.9%, and 100% in the number of mites in skin scraping samples on days 28, 56, and 84, respectively. Additionally, it was found that the decrease in mite numbers in dogs with generalized demodicosis treated with NexGard® was significantly higher compared to those treated with Advocate® spot-on (10 mg/kg imidacloprid, 2.5 mg/kg moxidectin, Bayer Animal Health GmbH) on days 28, 56, and 84 (89.8%, 85.2%, and 86.6% reduction, respectively). Four dogs naturally infected with *Demodex canis* and diagnosed with generalized demodicosis were treated with a dose of 2.5 mg/kg of afoxolaner (Oral, NexGard®, Boehringer Ingelheim Vetmedica GmbH) on day 0 and again at 4 and 8 weeks after the initial treatment. It was reported that live mites decreased in skin scraping samples and significant clinical improvement was observed in skin lesions after the treatment (99).

In a comprehensive clinical case evaluation series referred to a dermatology clinic, successful treatment of 102 cases of generalized demodicosis with afoxolaner was reported. Of the 102 patients, 68 were adult-onset demodicosis in dogs. Afoxolaner was initially administered at a dose of 2.5 mg/kg (oral) every two weeks to the first 10 dogs. Due to the high efficacy observed with the two-week interval administration, the frequency of administration was changed to once a month for the remaining cases. It was reported that 90% of the cases were negative for Demodex mites after two months of treatment, while the remaining dogs became negative after three months (100).

Lijima et al. (101) evaluated, afoxolaner's effectiveness in the treatment of demodicosis in dogs in a total of six cases, including five generalized and one localized case. Afoxolaner was given orally on day 0 and at intervals of 3 to 6 weeks at doses of 2.7-5.6 mg/kg body weight. Afoxolaner had to be administered once in two cases, twice in three cases, and three times in one case for the mites to be completely eradicated. In every case, it took 4–12 weeks after the initial

treatment for cutaneous lesions to disappear. Afoxolaner therapy has no side effects that were reported. Furthermore, no clinical relapses were observed in any of the cases within the first 6 months after the last examination.

Lebon et al. (84) conducted a multicenter study in France, Italy, and Poland to evaluate the effectiveness of izoxazoline group antiparasitic drugs containing afoxolaner (NexGard® or NexGard Spectra®) on 50 dogs with generalized demodicosis. Out of the 50 dogs included in the study, 31 were treated with NexGard® and 19 were treated with NexGard Spectra® orally, three times monthly. It was reported that there was a reduction of 87.6%, 96.5%, and 98.1% in mite counts on days 28, 56, and 84, respectively. In addition, itchiness, severity of skin lesions, and extent of skin involvement scores were significantly lower in all post-treatment evaluations compared to the baseline assessment. NexGard Spectra®, in addition to afoxolaner, contains milbemycin oxime. In this study, it was assumed that the short half-life of milbemycin oxime (2-3 days) would have no effect on the overall effectiveness against Demodex spp. Therefore, no comparison was made between NexGard® and NexGard Spectra®.

In a study conducted in Japan in 2018, a total of 15 dogs with generalized demodicosis, eleven of them with adult-onset and four with juvenile-onset, were treated with NexGard Spectra® for three months. NexGard Spectra® was administered orally at a dose of 2.5-6.3 mg/kg every four weeks. It was reported that there was a reduction of 91.2%, 99.8%, and 99.9% in mite counts on days 28, 56, and 84, respectively. Additionally, no adverse effects were observed following the treatment (102).

In a 2019 trial, the effectiveness of the drug combination afoxolaner + milbemycin oxime (NexGard Spectra®, Boehringer Ingelheim) for treating generalized demodicosis in dogs was assessed. The study included 68 dogs with generalized demodicosis in total. The dogs received a single oral dose of afoxolaner (2.50-5.36 mg/kg) and milbemycin oxime (0.50-1.07 mg/kg). The efficacy of the treatment was assessed based on visual evaluation of lesions and skin scrapings on days 7, 14, and 28 after treatment. It was reported that there was a significant reduction in lesions within the first 7 days, although the resolution of lesions varied. According to skin scraping samples taken after a single oral administration of NexGard Spectra®, the percentage of positive animals decreased from 100% on day 1 to 17.6% on day 28. This study indicated that the combination of afoxolaner with milbemycin oxime was effective in the treatment of generalized demodicosis in dogs and led to a reduction in lesions in the early days of treatment (103).

Generalized demodicosis was discovered in a 9-month-old female Thai mix breed dog with a history of skin conditions. On day 0 of treatment, the dog received afoxolaner (minimum 2.5 mg/kg, oral, single dosage). Following the treatment, a reduction in the size of the skin lesions was observed, and on day 14 after treatment, the hair started to regrow. Throughout the entire treatment, no adverse effects related to the medication were observed in the dog. During the 18-month follow-up period, no recurrence of clinical signs was observed. These findings suggest that single-dose oral afoxolaner can be considered as a beneficial treatment option for the management of generalized demodicosis (104).

In a study evaluating the clinical and bacteriological improvement in dogs with generalized demodicosis treated solely with acaricidal drugs without antibiotic or antiseptic therapy, four dogs with a diagnosis of pustular demodicosis based on skin scraping and cytology results were included. Two of these dogs were treated with a dose of 2.7-6.9 mg/kg afoxolaner on days 0 and 28. The other two dogs were treated with oral ivermectin at a dose of 0.5 mg/kg/24 hours for 63 days. Clinical scores were recorded on days 14, 35, and 56 after treatment, and cytology samples and smears were obtained from the skin lesions. Staphylococcal species were isolated from skin samples of all dogs on days 0 and 14 after treatment. However, cultures became negative in three out of four dogs on day 35 and in all dogs on day 56 after treatment. The preliminary findings from this study suggest that demodicosis-associated pyoderma in dogs can be resolved clinically, cytologically, and bacteriologically with a single acaricidal treatment while avoiding systemic antibiotics (105).

On the other hand, in a study conducted in 2017, the effectiveness of afoxolaner on Demodex populations in the skin of healthy dogs without a history of skin disease was investigated. Twenty healthy dogs were included in the study, divided into two groups: the afoxolaner group and the fluralaner group, with 10 dogs in each group. Throughout the 90-day study period, hair samples were collected from three different areas of the dogs' bodies on Day 0 (prior to treatment), Day 30, and Day 90. RT-PCR analysis was performed to amplify Demodex DNA in all samples. Five of the 20 dogs tested positive for Demodex DNA on Day 0 (before treatment), at least in one skin location (25%). Three of the 18 dogs tested positive on Day 60 (16.7%), and six of the 20 dogs tested positive on Day 90 (30%).In this study, it was reported that afoxolaner did not have a significant impact on Demodex populations in the skin flora of healthy dogs throughout the 90-day period (106).

In a parallel-group, double-blind, randomized, single-center, negative-controlled efficacy study conducted in 2018, the effectiveness of oral treatment with chewable tablets of NexGard® or NexGard Spectra® was evaluated in eight dogs diagnosed with generalized demodicosis. The study was conducted on three groups of eight dogs each: Group 1 dogs were untreated controls; Group 2 dogs were treated orally with NexGard® chewable tablets; and Group 3 dogs were treated orally with a combination of NexGard Spectra® chewable tablets. Dogs in Group 2 were treated with a therapeutic dose of afoxolaner (as close to 2.5 mg/kg as possible), and dogs in Group 3 received afoxolaner (as close to 2.5 mg/kg as possible) in combination with milbemycin oxime at a dose of 0.5 mg/kg on Days 0, 28, and 56. From Day 7 through Day 84, general health inspections were performed once each day on all dogs. Three NexGard or NexGard Spectra monthly treatments were proven to be extremely efficient against canine generalized demodicosis, with 99.9% and 100% efficacy against mites, respectively. Both treatments resulted in a significant reduction of skin lesions and over 90% hair regrowth three months after the initial treatment (107).

Based on above data, afoxolaner can be considered an effective, safe, and suitable treatment option for dogs with generalized demodicosis.

4.2. Fluralaner

Fluralaner (Bravecto™, MSD) is an acaricide and insecticide that is effective against ticks (*Ixodes* spp., *Dermacentor* spp., and *Rhipicephalus sanguineus*) and fleas (Ctenocephalides spp.) in dogs. (108,109,110,111) Fluralaner is also used in the treatment of demodicosis and sarcoptic mange. (60,112,113) This active ingredient is rapidly absorbed after oral administration. It reaches its maximum concentration within 24 hours and remains detectable in the plasma for up to 112 days. (114) Fluralaner exhibits high potency against ticks and fleas that are exposed through oral ingestion, meaning it has a systemic effect on the target parasites. Fluralaner acts as an antagonist on ligand-gated chloride channels (GABA receptor and glutamate receptor), resulting in a strong inhibitory effect on various parts of the arthropods' nervous system (108,109).

Fourie et al. (60) conducted a study comparing the effectiveness of Advocate® formulated for topical application (10 mg imidacloprid/kg body weight and 2.5 mg moxidectin/kg body weight, topical, 3 applications at 28-day intervals, n=8) and Bravecto™ formulated as a chewable tablet (fluralaner, 25 mg/kg, oral, single dose, n=8) for the treatment of naturally infected generalized demodicosis in dogs. The results showed that Bravecto™ chewable tablets, after

a single oral dose, resulted in clinical improvement and no mites were detected in skin scrape samples taken on days 56 and 84, indicating the effectiveness of fluralaner against generalized demodicosis. On the other hand, in the other treatment group, Advocate® administered three times at 28-day intervals was found to be effective against generalized demodicosis, but it had the disadvantage of still showing demodex mites in skin scrape samples taken on days 28, 56, and 84 in most dogs.

In an another clinical study conducted in 2015, 163 dogs with generalized demodicosis were treated with Bravecto® (25 mg/kg fluralaner, oral). At the end of the first month, 87% of the 163 dogs had achieved negative skin scrapings, and by the end of the second month, 100% of them had obtained negative results. Furthermore, no adverse effects were recorded following the treatment (112).

Four dogs diagnosed with demodicosis were treated with two doses of oral fluralaner (25 mg/kg, Bravecto™, MSD) at a 60-days interval. It was reported that the number of mites decreased by 98% on the 90th day after treatment. Additionally, no treatment-related side effects were recorded (115). Similarly, in a case report presented by Benito et al. (116), a dog diagnosed with demodicosis caused by Demodex injai was treated with a single dose of oral fluralaner (25 mg/kg, Bravecto™, MSD). It was stated that on the 49th day after treatment, the dog was completely free of mites (100% mite-free). Additionally, no side effects were recorded following the treatment.

In a study conducted in 2017, the efficacy of fluralaner on Demodex populations in the skin of healthy dogs without a history of skin diseases was investigated. Twenty healthy dogs were included in the study, with 10 dogs in each group (afoxolaner group and fluralaner group) for a duration of 90 days. Prior to drug application on Day 0, and then on Days 30 and 90, hair samples were collected from three areas of the body. On each sample, Demodex DNA was amplified using RT-PCR. Day 0 tests revealed that Demodex DNA was present in five of the 20 canines (about 25%) in at least one skin location. Three out of 18 dogs tested positive on day 60 (16.7%), and six out of 20 dogs tested positive on day 90 (30%). The study reported that fluralaner did not have a complete effect on Demodex populations in the skin flora of normal dogs over the 90-day period (106).

Duangkaew et al. (117), evaluated the effectiveness of oral fluralaner (Bravecto™) in the treatment of generalized demodicosis in dogs at a dose of 25-50 mg/kg. In the study, there were 115 dogs, 73 of which had adult-onset demodicosis and 42 of which had juvenile-onset demodicosis. After treatment,

48 dogs were excluded from the study due to various reasons, and 67 dogs (73 with adult onset demodicosis and 42 with juvenile onset demodicosis) were successfully treated with a fluralaner. It was reported that in adult onset demodicosis, improvement was observed in 63%, 85%, and 100% of the dogs at two, three, and four months, respectively. Similarly, in juvenile onset demodicosis, improvement was reported in 81% and 100% of the dogs at two and three months, respectively. The application of fluralaner at the recommended dose for flea and tick prevention was found to be effective in the treatment of generalized demodicosis in dogs. Additionally, none of the dogs showed any fluralaner adverse effects.

Morita et al. (88) reported the effectiveness of a single dose of oral fluralaner in a concurrent refractory generalized demodicosis case in a 12-year-old female Shih-Tzu dog previously diagnosed with hyperadrenocorticism and hypothyroidism. In this case, amitraz treatment was effective, but discontinuation of amitraz and trilostane therapy resulted in the development of diabetes. Attempts to control diabetes were unsuccessful, and hyperadrenocorticism was left untreated, leading to a relapse of demodicosis. However, demodicosis was successfully treated with a single dose of fluralaner (Bravecto™, MSD). Transient erythematous papules were observed on the body three days after fluralaner application, but no other side effects were recorded. The study reported that fluralaner is a potent and effective treatment for demodicosis and that skin rashes can be observed after the initial dose of the medication.

In a laboratory study conducted in 2019, the effectiveness of two topical spot-on treatments, fluralaner (Bravecto™ Spot-on Solution, Merck Animal Health, Madison, NJ, USA) and the combination of imidacloprid and moxidectin (Advocate®, Bayer Animal Health, Leverkusen, Germany), were compared in naturally infected dogs with generalized demodicosis. Sixteen dogs were included in the study and randomly assigned to one of the two study groups, each consisting of eight dogs. On Day 0, dogs in Group 1 were treated once with fluralaner spot-on solution. Dogs in the other group received imidacloprid/moxidectin spot-on solution either three times (on Days 0, 28, and 56) or weekly in severe cases. Demodex mites were counted in skin scrape samples and skin lesions were evaluated before treatment and at 28-day intervals for a duration of 12 weeks. After fluralaner application, the miticidal efficacy was determined to be 99.7% on Day 28, >99.9% on Day 56, and 100% on Day 84. In dogs treated topically with imidacloprid and moxidectin combination, the miticidal efficacy was determined to be 9.8% on Day 28, 45.4% on Day 56, and 0%

on Day 84. The efficacy was significantly lower ($P<0.01$) at all time points compared to the group treated with fluralaner. A single topical application of fluralaner successfully eliminated Demodex spp. mites in dogs with generalized demodicosis. However, topical treatment with imidacloprid and moxidectin combination administered at 28-day intervals three times or more frequently was unable to completely eliminate the mites in most treated dogs (70).

In another study conducted in Brazil in 2019, the effectiveness of fluralaner (25 mg/kg, oral, single dose) was investigated in 15 dogs diagnosed with generalized demodicosis. Two out of the 15 included animals were excluded from the study because they did not return for the second follow-up examination within 60 days, despite having an initial negative skin scraping. Among the 13 dogs that completed the study, six of them (46.2%) showed the first negative skin scraping at the end of the first month. At the end of the study, all dogs except three were reported to have recovered. Therefore, oral single-dose fluralaner was found to be effective in the treatment of generalized demodicosis in dogs (118).

In order to identify and measure DNA from Demodex spp., Djuric et al. (119) used real-time polymerase chain reaction (RT-PCR) to investigate the efficacy of fluralaner in the treatment of generalized demodicosis in dogs. They included 20 owned dogs who had Demodex canis mites in their deep skin scrapings and had clinical signs of generalised demodicosis. Each dog was given with fluralaner at the dosages (25–56 mg/kg) suggested for tick and flea control based on body weight after diagnosis (Day 0). In skin scrapings taken from all canines, a single oral dose of fluralaner reduced the number of Demodex mites by 98.9% on Day 28 and 100% on Day 56. Additionally, On Day 56 after treatment, complete regrowth of hair and clinical improvement were reported in the dogs.

In a field study conducted in Europe in 2020, the efficacy of fluralaner (Bravecto®, spot-on and chewable tablets, oral, single dose) and topical imidacloprid + moxidectin (Advocate®, monthly or weekly) combination therapy was compared in 134 dogs diagnosed with generalized demodicosis. Fifty-seven dogs had juvenile-onset demodicosis and 67 had adult-onset demodicosis out of the 124 dogs that completed the research. Oral or spot-on administration of fluralaner was found to be effective, reducing the number of Demodex mites in at least 98.0% of the dogs on days 56 and 84. In comparison to adult-onset demodicosis, oral and spot-on formulations were observed to be 100% and 96.7% effective against juvenile-onset demodicosis, respectively. A single

application of chewable or spot-on fluralaner was found to be highly effective against both forms of generalized demodicosis. No treatment-related side effects were observed. Imidacloprid-moxidectin treatments given repeatedly, on the other hand, were less successful, removing mites from only 87.5% of the dogs (92.0% in cases of juvenile-onset demodicosis and 81.8% in cases of adult-onset demodicosis). The topical application of imidacloprid-moxidectin combination over an 84-day period did not achieve completely successful results (mite-free skin) (120).

Vargo and Banovic, (121) an 11-year-old neutered male Shetland Sheepdog was brought to the clinic due to a history of localized, non-itchy alopecia and scaling affecting the peri-nasal region, which had started three weeks prior as a result of long-term inhalation glucocorticoid (fluticasone) treatment for chronic bronchitis. The dog was diagnosed with localized demodicosis (*Demodex canis*). The dog was then given oral fluralaner as a treatment. After 8 weeks of treatment, the demodicosis clinical indications improved, and the trichogram results revealed no living or dead Demodex mites.

A multicenter (conducted in 9 veterinary clinics), prospective study was conducted by Hoshino et al. (122) to evaluate the effectiveness of oral fluralaner in the long-term (>12 months) treatment of demodicosis in dogs. A single dose of oral fluralaner was administered to the dogs included in the study. Each dog received thorough parasitological and dermatological examinations every three months over a period of more than a year. Out of the 26 dogs included in the study (age: 3 months - 16 years), 9 had juvenile onset, 17 had adult onset, and 18 had generalized demodicosis, while 8 had localized demodicosis. The administration of a single dose of fluralaner resulted in the complete eradication of mites in all dogs within three months and the resolution of all skin lesions. Seventeen dogs were either required a second dose of isoxazoline or were excluded for reasons unrelated to fluralaner treatment, meaning they were not included in the one-year follow-up assessment. None of the nine remaining cases—six with adult onset and three with juvenile onset; six with generalized and three with localized form—seen any of the dogs experience relapses. The results of this study show that, when paired with the control of underlying diseases, a single dosage of fluralaner can successfully offer long-term therapy.

A 10-year-old neutered male Shih-tzu dog was diagnosed with treatment-resistant generalized demodicosis that persisted for one year despite traditional ivermectin and amitraz treatment (123). The dog was concurrently diagnosed with deep pyoderma, Malassezia dermatitis, and otitis externa. A treatment

regimen consisting of amoxicillin-clavulanic acid, antifungal drugs (itraconazole, miconazole), and milbemycin oxime resulted in a favorable response for 90 days. However, approximately 4 months later, the first recurrence of demodicosis occurred, and the miticidal treatment was changed to ivermectin. Despite continuing the 440-day ivermectin treatment, a second recurrence occurred, and amitraz baths were added to the treatment. However, the demodicosis persisted in the dog, and rapid clinical improvement was reported when the miticidal treatment was switched to oral fluralaner. Additionally, it was noted that no further relapses occurred approximately 920 days after the administration of oral fluralaner before the dog's death. This case report highlights the successful use of oral fluralaner in cases of recurrent and persistent demodicosis.

Sree Krishna Sai et al. (124) investigated, the effectiveness of fluralaner in the treatment of generalized demodicosis in dogs that they treated 6 out of 9 dogs diagnosed with demodicosis with oral fluralaner (Bravecto™) at a dosage of 30 mg/kg body weight as a single dose. The dogs also received antibiotic treatment (cephalexin, 15 mg/kg body weight, twice daily for one week), benzoyl peroxide shampoo, and supportive care. After four weeks of treatment, complete clinical improvement was observed in the included dogs, and consecutive skin scrape samples were reported as negative. The findings of this study indicate that fluralaner achieved 100% recovery in the treatment of generalized demodicosis, and no adverse effects were recorded during the clinical evaluation.

In a study conducted in 2022, the efficacy of a new fluralaner chewable tablet formulation (%5.46 w/w) for monthly application in the treatment of generalized demodicosis in dogs was investigated. The dogs included in the study were randomly divided into two groups: one group received fluralaner chewable tablets (Bravecto® 1-Month, %5.46 w/w, minimum dose rate of 10 mg/kg body weight), and the other group received topical imidacloprid-moxidectin (Advocate®, Elanco). On the 28th day, it was found that the pre-treatment mite counts significantly decreased by 99.7% in the fluralaner group and 89.5% in the imidacloprid-moxidectin group. On the 56th and 84th days, all dogs treated with fluralaner showed 100% parasitological improvement (100% reduction in mite count). In the imidacloprid-moxidectin group, the average mite count reduction was determined as 89.5% (28th day), 94.4% (56th day), and 97.5% (84th day). The complete recovery rate in dogs treated with imidacloprid-moxidectin was found to be 25%. These findings indicate that the new fluralaner chewable tablet formulation (%5.46 w/w) can provide effective results in eliminating *D. canis* mites in dogs diagnosed with generalized demodicosis (125).

Eighteen demodicosed dogs and 6 healthy dogs were included in a trial to determine fluralaner's efficacy in treating demodicosis in dogs. The dogs were divided into 4 groups, with 6 animals in each group: Group 1 (Control), Group 2 [Amitraz %12.5 (0.05%, topical, once a week, for 4 weeks) + ivermectin (0.2 mg/kg, once a week, for 4 weeks, SC)], Group 3 [Saffron oil (topical, for 15 days) + Saffron tablet (for 10 days, PO)], and Group 4 [Fluralaner tablet (25 mg/kg, single dose, PO)]. The dogs treated with fluralaner showed a decrease of 61.72% and 91.18% in mite numbers on days 15 and 30 post-treatment, respectively, along with clinical improvement. Microscopic examination of deep skin scrapings from 3 dogs in Group 4 revealed no mites, and it was reported that all dogs showed a rapid but slow clinical improvement compared to those in Group II (25).

Based on above data, fluralaner can be considered an effective, safe, and suitable treatment option for dogs with generalized demodicosis.

4.3. Lotilaner

Lotilaner is the newest active compound among the isoxazoline class. (126-128) Lotilaner is available in the market as both a standalone product (Credelio®, Elanco) and in combination with milbemycin oxime (Credelio® Plus, Elanco). (129,130) Lotilaner is used as an ectoparasitic agent in the treatment of sarcoptic mange, demodicosis, tick, and flea infestations in dogs (14,86,129,131-134). In a study conducted in 2017, it was found that lotilaner, similar to other active compounds in the isoxazoline group, causes inhibition of γ-aminobutyric acid (GABA) channels in the central nervous system, which acts as a neurotransmitter. This inhibition leads to the entry of chloride ions into the cells, resulting in hyperpolarization and ultimately leading to the death of parasites (126). Lotilaner (Credelio™) is formulated as a flavored chewable tablet. It is rapidly absorbed and reaches its peak blood concentrations within 2 hours after treatment. Lotilaner has an approximate half-life of 30 days, which means insecticidal and acaricidal blood levels are maintained for at least 1 month after treatment (128).

Milbemycin oxime and lotilaner-flavored chewable tablets' acute and long-term safety in healthy beagles were investigated in three randomized, blinded, parallel-group investigations. Thirtytwo dogs were randomly assigned to one of four groups in each of the two long-term studies—either untreated controls or groups that received the 1X, 3X, or 5X target doses—in each study. Milbemycin oxime and lotilaner were found to have no treatment-related adverse effects

in terms of health observations, physical/neurological examinations, or food consumption in long-term or acute studies. Additionally, it was noted that in long-term investigations, these substances had no treatment-related impacts on clinical pathology, body weight, or macroscopic and microscopic exams. The results of this study show that monthly administration of Credelio® Plus to pupies and dogs at the upper limit of the commercial dose band is safe (130).

In a study conducted in 2017, the safety of Credelio® chewable tablets was evaluated in eight-weeks-old beagle puppies. Over a period of eight months, 16 female and 16 male puppies, aged four weeks, were treated with lotilaner (20-43 mg/kg, Credelio® chewable tablet, Elanco). The puppies included in the study were divided into four different groups, including one control group. The dogs in the study groups were administered Credelio® tablets at doses of 1, 3, and 5 times the maximum recommended dose by the manufacturer (43 mg/kg, 129 mg/kg, and 215 mg/kg body weight), respectively. Throughout the study, the dogs were clinically examined, including blood parameters and urine analysis, as well as neurologic and ophthalmologic evaluations. It was reported that Credelio® had no effect on body weight or food consumption and had no adverse effects on physical, neurological, or electrocardiographic parameters (127).

In an another study conducted in 2017, in dogs naturally infected with Demodex spp. and identified as having generalized demodicosis, the oral systemic effectiveness of Lotilaner (Credelio®, Elanco) was assessed (129). The study included 10 dogs with generalized demodicosis, and they were treated with oral Lotilaner (at a minimum dose of 20 mg/kg) on days 0, 28, and 56. It was reported that the mite counts in the dogs treated with Lotilaner decreased by >99.9% from the pre-treatment counts to the 56th day after the first and second monthly doses. Significant improvement in clinical signs of demodicosis was observed in all dogs treated with Lotilaner, and there have been no recorded drug-related side effects.

Based on above data, Lotilaner can be considered an effective, safe, and suitable treatment option for dogs with generalized demodicosis.

4.4. Sarolaner

Sarolaner is a new ectoparasitic active compound from the class of isoxazolines. (92,135,136) In the market, Sarolaner is available as Simparica® Chewable Tablet (Sarolaner, Zoetis), Simparica TRIO® Chewable Tablet (Sarolaner, Moxidectin, and Pyrantel, Zoetis), and Revolution Plus® (Selamectin and Sarolaner, Zoetis). These products are sold as both single and combination

formulations (Six et al., 2016; Becskei et al., 2018). (137,138) Sarolaner is an ectoparasitic agent used in dogs as a monthly oral dose acaricide and insecticide against fleas, ticks, and mites (135,137-144). In addition, it has been reported by numerous researchers that sarolaner is also effective in regard to sarcoptic mange therapy, otodectic mange, and demodicosis in dogs, with successful outcomes (89,90,137,138).

Following oral treatment, sarolaner is quickly and effectively absorbed and enters the systemic circulation. Thanks to this feature, it reaches its maximum plasma concentration within the first 24 hours after administration. Sarolaner has been found to be safe in dogs 8 weeks and older when administered repeatedly monthly dosages of up to 20 mg/kg. The bioavailability of sarolaner has been calculated to be >85%, and this compound exhibits high plasma protein binding (>99.9%). The half-life of sarolaner is estimated to be 11-12 days. After oral administration to dogs, sarolaner plasma concentrations have demonstrated dose proportionality in the range of 1.25-5 mg/kg, and these same doses have shown strong efficacy (≥99%) against both fleas (*Ctenocephalides felis*) and many tick species (*Rhipicephalus sanguineus, Ixodes ricinus, and Dermacentor reticulatus)* for ≥35 days (135).

Research was done by Becskei et al. (138) to determine if sarolaner is effective for treating dogs with generalized demodicosis. The dogs included in the study were divided into two groups. Dogs in Group 1 were treated monthly with oral sarolaner (Simparica®, Chewable Tablet, Zoetis, USA) (n=53). The dogs in Group 2 (n=28) received topical moxidectin/imidacloprid (Advocate®, Spot On Dog, Bayer, Leverkusen, Germany) therapy either weekly or monthly, based on the severity of the lesions. It was found that after three to five months of treatment with sarolaner, 92.9% and 100% of the dogs achieved healing, respectively. After three and six months, 77.3% and 91.7% of the dogs in the moxidectin/imidacloprid group, respectively, had recovered. When comparing the mite counts of the dogs in the sarolaner group with the pre-treatment counts, it was determined that they decreased by 77.2%, 95.0%, 98.5%, 99.0%, 100%, and 100% on days 30, 60, 90, 120, 150, and 180, respectively. In the treatment of dogs with generalized demodicosis, sarolaner is reportedly risk-free, extremely effective, and free of negative side effects. Six et al. (143), conducted a study to evaluate the efficacy of sarolaner (Simparica™, Zoetis) in the treatment of generalized demodicosis (*Demodex* spp.) and otodectic mange (*Otodectes cynotis*) in dogs. A total of sixteen canines with clinical indications of generalized demodicosis were treated orally with sarolaner (2 mg/kg, Oral,

Simparica™, Zoetis, USA) or topically with imidacloprid + moxidectin solution (Advocate®/Advantage® Multi Spot-on solution, 100 mg imidacloprid + 25 mg moxidectin/ml, Bayer) on days 0, 30, and 60. It was found that in dogs treated with sarolaner, there was a reduction of 97.1% in mite counts 14 days after the first dose and 99% reduction after 29 days, with no further detection of live mites. In dogs receiving weekly imidacloprid + moxidectin treatment, mite counts were reduced by 84.4% and 95.6% at these two time periods, respectively, and no mites were found after day 74. In this study, an oral dose of sarolaner at 2 mg/kg was highly effective in reducing the number of live mites associated with *Demodex* spp. Dogs treated with sarolaner showed significant improvement in clinical signs of generalized demodicosis. Additionally, no treatment-related side effects were reported.

Sarolaner's effectiveness in treating six dogs with widespread demodicosis was assessed by Prins et al. (89). Sarolaner chewable tablets (2 mg/kg body weight, single dosage, SimparicaTM, Zoetis, USA) were administered to the dogs. After one week of treatment, all dogs showed rapid clinical improvement, and complete recovery was reported after one month. Additionally, it was reported that there were no treatment-related side effects in any of the cases.

A 4-month-old male Boxer weighing 10 kg, diagnosed with demodicosis (*Demodex canis*), was treated with a combination of ivermectin, a shampoo containing benzoyl peroxide, amitraz (spray), and cephalexin for a duration of 24 days until complete recovery and a negative skin scraping report for demodicosis. (90) However, one week after the completion of treatment, the dog experienced a recurrence of itching and redness on the skin. In response, the case was treated with Sarolaner (Simparica®, Zoetis, USA). The treatment was repeated four times at 35-day intervals. It was reported that the dog completely recovered within 5 days after treatment with Sarolaner and did not experience any relapses in the following period.

A combination therapy of Sarolaner (4 mg/kg body weight, oral, single dose, Simparica®, Zoetis) with a biostimulant (Catosal, 100 ml: butafosfan (10 g), cyanocobalamin (0.005 g), methyl 4-hydroxybenzoate (0.1 g), intravenous, 3 ml, once daily for 5 days, Bayer HealthCare LLC, USA) and a hepatoprotective agent (3 ml, intravenously, every day for five days, Tioprotectin, Arterium, Ukraine) was evaluated for its therapeutic efficacy in dogs with demodicosis. (145) Different treatment procedures on dogs of various genders, ages, and breeds with generalized demodicosis form were used to investigate the efficacy of Sarolaner. This study demonstrated that Sarolaner (Simparica®, Zoetis)

alone is highly effective against demodicosis in dogs, even in a single dose. However, the efficacy of Sarolaner as monotherapy decreased to 71.4% and 57.1% in pustular and mixed clinical forms of demodicosis, respectively. When Sarolaner was combined with Catosal in pustular and mixed demodicosis forms, the therapeutic efficacy increased to 85.7%. When a hepatoprotective agent (Thioprotectin) was added to this dual treatment combination, the therapeutic efficacy was reported as 100% and 85.7% in pustular and mixed demodicosis forms, respectively. The findings from this study suggest that a combination of Sarolaner (Simparica®, Zoetis) with Catosal and Thioprotectin would lead to better results in the treatment of generalized demodicosis in dogs.

In a 2019 trial, oral Sarolaner at a dosage of 2.0 mg/kg every 30 days was administered to 27 dogs with generalized demodicosis. On days 0, 30, 60, and 90, sarolaner was given. The canines were evaluated to see whether they had demodicosis. Every 30 days for 90 days, clinical data were collected both before and after the therapy. Skin scraping tests for demodicosis were negative in 77.7% of the dogs on day 30, and parasitological testing was negative in 100% of the dogs on day 60. This trend persisted until day 90. 7.41% of the dogs displayed clinical remission on day 30, and 74.07% did so on day +60. Day +90 revealed that 88.88% of the canines had fully recovered clinically. According to this study, Sarolaner is a highly successful alternative to standard therapy for dogs with generalized demodicosis, particularly in situations when ivermectin is contraindicated (146). Based on above data, Sarolaner can be considered an effective, safe, and suitable treatment option for dogs with generalized demodicosis.

5. Conclusion

As a result, afoxolaner, fluralaner, lotilaner and sarolaner are active substances belonging to the isoxazoline group that have been widely used in recent years. The use of isoxazoline group drugs has become a popular choice in the treatment of demodicosis in recent years due to its ease of use, fewer adverse effects than other drugs, and its effectiveness in stubborn cases that do not respond to traditional treatments (6,14,86).

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CHAPTER IV

THE LATEST INTRACELLULAR CARGO MEMBER: EXOSOMES

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1. Introduction

In multicellular creatures, billions of cells must cooperate to carry out multiple tasks at once. Short-range cell communication is mediated by neurotransmitters and local regulators. Extracellular vesicles and hormones both contribute to long-distance communication. Extracellular vesicles are divided into three groups: apoptosome, micro vesicle, and exosome, according to their size and formation (1).

One of which is the exosome, which is a natural vesicle released by cells into the extracellular environment and varies in size between 40 and 100 nanometers. These vesicles are surrounded by a double-layered phospholipid membrane (2).

Exosomes are formed inside multivesicular bodies within the donor cell by invagination of their membrane. A complex mechanism then loads a series

of specific molecules into the exosome as cargo. The MVB is then transported to the plasma membrane, where it fuses to release the exosomes, it contains into the "extracellular space." (3).

The majority of the cargo carried by exosomes consists of epigenetic material (protein transcription factors, a wide range of RNAs, and DNA lengths). Epigenetic material consists of four main categories: (a) molecules that act directly on DNA by promoting covalent binding (e.g., DNA methylases and demethylases) or noncovalent binding (e.g., protein transcription factors); (b) substances that modulate the accessibility of DNA by promoting covalent binding to histones (e.g., histone methylases and acetylases); (c) mRNAs that induce de novo protein synthesis in the target cell; and (d) microRNAs that bind to mRNAs and modulate their activity (4).

Exosomes were first isolated from cultured cells but were subsequently proven to be released from many cells, including red blood cells, platelets, epithelial cells, lymphocytes, dendritic cells, and tumor cells (5). Exosome cargo is regulated by the immune status of the cells forming the vesicles. Transport of exosomes is carried out to distant points through cell-cell contact or by transfer across gap junctions or synapses (4).

Exosomes find the right target cell through different glycosylation patterns on their surfaces that can serve as identifying signals. (6) Thus, the exosome and the target cell recognize each other and bind to complementary glycosylation patterns on them. Another possible recognition molecule is heparin sulfate proteoglycans (HSPGs), and exosomes can also enter cells via HSPG-mediated endocytosis. Heparanase enzyme activity is required for strong enhancement of exosome secretion (7,8).

1. Clinical Importance of Exosomes

Exosomes have lower immunogenicity compared to liposomes or viral vectors, and their ability to cross important physiological barriers such as the blood-brain barrier makes them an attractive and innovative option as biomarkers and therapeutic agents (9).

In recent years, with the understanding of the important physiological properties of exosomes and their important roles in health and disease, interest in the potential clinical applications of these structures for the diagnosis and treatment of diseases has been rapidly increasing (2).

Exosomes are better tolerated by the body because they are formed by all body cells, resemble cell membranes in structure and content, and can pass

through complex barriers such as the blood-brain barrier. Their role in the transport of bioactive compounds with low solubility and bioavailability attracts attention. Exosomes are present different biological fluids such as serum, plasma, urine, saliva, ascites, cerebrospinal fluid, and amniotic fluid (1).

Another important fact is that exosomal lipoprotein vesicular coatings can protect the exosome cargo against catabolic enzymes found in phagolysosomes (10).

Exosomes are released in pathophysiological situations such as inflammation, neurodegeneration, immune response, cancer, cell death, or angiogenesis. Because they contain structural components, including transmembrane proteins or nucleic acids, they can be used as biomarkers for clinical diagnosis, staging of disease severity, or assessment of therapeutic response (11).

2. Exosomes Related to Food

Exosomes have been isolated from foods such as lemon, ginger, and milk. The potential role of food-derived exosomes (FDEs) in alleviating diseases as well as modulating the gut microbiota has been demonstrated in both babies and adults (12).

Nutrition is one of the environmental factors affecting microbiota composition and function. Food-derived compounds promote gut health either directly or by modulating the composition and function of the gut microbiota and interacting with factors and/or signaling pathways associated with immune function (13).

The functionality of extracellular vesicles with this biological function and the particles they carry are the basic mechanisms behind the harmful or beneficial effects of many pathogenic, symbiont, or probiotic bacteria (14).

For example, lemon exosome-like nanoparticles (LELNs) have been shown to inhibit the death of apoptotic cells, which suppresses cancer cell proliferation (15,16), or to inhibit the growth of colorectal cancer cells brought about by p53 inactivation (17).

miRNA, along with membrane-integrating lipids and other bioactive substances present in the exosome payload, may play a crucial role in the interaction between plant exosomes, also known as diet-related nanoparticles, and bacteria. These substances influence the regulation of the microbiota in a favorable or negative way (18).

In addition to their effects on the microbiota, food-derived exosomes also directly affect the host. These exosomal miRNAs can be absorbed through the intestine and play important functions in the organism. Exosomes generated from ginger were found to aid in the repair of mucosal tissues in mice with colitis, according to a study (19).

More importantly, miRNA-containing exosome vesicles are found in milk, and they contain rich immune-related miRNAs (20). In a similar study, it was discovered that exosomes can be found in cooked meat products, including pork muscle, fat, and liver, and that there may be variations in exosomal miRNA between tissues. Mice fed exosomes produced from cooked pork also developed insulin resistance and abnormalities in hepatic lipid metabolism (21).

Additionally, it is claimed that food-borne exosomes can affect conditions of the neurological system, skeletal-muscular system, nervous system inflammation, and cancer in different ways (12).

3. The Relationship Between Exosome and Microbiome

Exosomes involved in this communication may originate from food as well as directly from the microbiome. Bacterial cells often interact with their hosts and other bacteria directly and secrete soluble substances and extracellular vesicles (EVs) to communicate (22,23).

Exogenous miRNAs from the diet and endogenous miRNAs from the host are both present in these vesicles (EV, the newly discovered forerunner of intercellular communication). Studies have demonstrated their role in mediating intercellular communication, although exosomes were originally thought to be cellular waste disposal mediators. (24). Enteric bacteria (both pathogenic and commensal) derive EVs to communicate with their hosts, and these spherical membrane-encapsulated particles transfer some biological components of the parent bacteria to the extracellular environment (25,26).

There is growing evidence that the gut microbiome, the human body's modifiable "second genome," is a critical determinant of human health and disease. The genetics and nutrition of the host play a key role in shaping the microbial composition (27).

The interaction between the immune system and the more than 100 trillion commensal microorganisms (bacteria, archaea, fungi, and protozoa) living in the gastrointestinal tract, numbering approximately 100 trillion times more than the total number of cells in the human body, is the focus of many studies (28,29).

Extracellular vesicles have recently been discovered to exhibit particular modulatory properties of the gut microbiota (27).

The majority of EVs are released as membrane vesicles (MVs) and outer membrane vesicles (OMVs) by gram-positive and gram-negative bacteria, respectively (25). OMVs boost virulence by interacting with immune system mediators and producing cytotoxic factors in host cells, according to studies, particularly on Gram-negative pathogens (30,31). As a result, it is conceivable that microbiota vesicles may play a key role in the signaling process of the intestinal mucosa (31).

The intestinal microbiota and the function of distant cells can be modulated through the apical secretion of exosomes into the lumen and the transport of antimicrobial products from the gastrointestinal tract (32).

EVs from the microbiota are always concentrated in tissues like the gastrointestinal tract that are in close contact with bacteria. Therefore, gut microbial-released EVs, which contain various RNA species (e.g., mRNA, miRNA, and tRNA) and have multiple functions, are biologically active components and can influence host gene expression (33-35).

EV derived from each bacteria induces different physiological responses (36). For example, EV from the bacterium Akkermansia muciniphila has been linked to alleviating high-fat diet-induced obesity and diabetes in mice. They also included inhibiting the intestinal barrier disrupting, lowering weight loss, and managing the inflammatory response (37).

4. Other Uses of Exosomes

Apart from the above-mentioned functions, other current studies on exosomes are frequently carried out on their use in carrier cargo and vaccine production. A study on the utilization of milk-derived exosomes to boost antioxidant absorption and stop their deterioration in the GIS, particularly after oral ingestion, came to the conclusion that they could serve as a cargo for new complementary medicines or functional foods (38).

Extracellular vesicles (EVs) facilitate intercellular communication and can significantly amplify the immune response by delivering antigens, so using exosomes—which transfer antigens from SARS-CoV-2 structural proteins—to generate a SARS-CoV-2 vaccine has been put on the spotlight (39).

A novel respirable virus-like particle-containing vaccine has been planned against the COVID-19 pandemic, based on the integration of the SARS-

CoV-2 receptor-binding domain and lung-derived exosomes. Lung-derived exosomes served as an excellent vehicle that effectively delivered the vaccine to the targeted site and was retained in the lung for a longer period of time for subsequent vaccination. It has been demonstrated that such an inhalable vaccine can effectively resist viral infection, reduce lung lesions, and trigger a strong T-cell immune response by triggering strong mucosal and cellular immunity (40).

5. Conclusion

Considering food safety from field to table, plant exosomes/nanoparticles are extremely important both in human nutrition and in the nutrition of animals offered for human consumption. On the other hand, it has been demonstrated that miRNAs found in exosomes, especially those obtained from meat and dairy products consumed as animal foods, significantly affect the immune response. Exosomes from food interact directly with the intestinal cells of the host or indirectly through the host microbiota to influence the immune response. The performance of new studies on the topic will help researchers better understand the pathophysiology of the many autoimmune and metabolic disorders that are prevalent today. On the other hand, the use of exosomes for drug and vaccine production will shed light on the production of cargo molecules that can reach the target cell faster and have fewer side effects.

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CHAPTER V

THE ROLE OF APOPTOSIS IN THE PATHOGENESIS OF VIRAL DISEASES

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1. Introduction

Apoptosis has been found to function as an important defense mechanism against viral infections in multicellular organisms. When infected host cells are destroyed by apoptosis, viral replication cannot occur, and thus the defense system of the host exerts a destructive effect on the virus infection. However, most viruses have developed various strategies to prevent apoptosis of the host cell (1).

Eukaryotic cells die by necrosis or apoptosis. There are morphologic, biochemical and physical differences between these two forms of cell death (2, 3).

Apoptosis, quite unlike necrosis, is both an energy-demanding and genetically controlled process. During apoptosis, unlike necrosis, the cell shrinks and loses 30% of its volume in less than an hour. While mitochondria are morphologically intact in the apoptotic cell, the main target damaged is the cell nucleus (4). In severe cases, apoptosis may be accompanied by a large number of necrotic cells. This suggests that apoptotic stimuli triggered by the virus lead to cell death by necrosis in some infected cells. The ratio of these two types of cell death to each other depends on the physiologic state of the infected cells (5).

There is a strong relationship between induction of apoptosis and suppression of viral replication. It has been reported that apoptosis alone can inhibit the development of viral replication (6). Many factors within apoptotic pathways are responsible for the antiviral reaction in infected cells and are also involved in macromolecule synthesis regulation (7,8,9). Among these,

antiapoptotic gene products of the virus enable virus proliferation by inhibiting apoptosis. In invitro studies with HSV-1 (DNA virus), VSV (RNA virus) and poliovirus (RNA virus), it was observed that even in the presence of sorbitol, which has apoptosis-inducing properties, these viruses were able to realize viral proliferation by suppressing apoptosis thanks to their antiapoptotic genes (10,11,12). These studies revealed that apoptosis has an important role in the pathogenesis of viral diseases (13,14,15).

During a viral infection, viruses normally make the host cell synthesize their own proteins in the infected cell and stop the cell from making proteins necessary for the cell itself. Thus, apoptosis is induced in the infected cell and the cell dies. In this way, the virus also destroys itself. However, some viruses (e.g., Ebstein-Barr virus or human papillomavirus) have developed ways to suppress apoptosis in the infected cell (15). Among these viruses, Ebstein-Barr virus prevents apoptosis by producing molecules similar to Bcl-2, one of the regulators that control the induction of apoptosis, and by producing molecules that enable the infected cell to synthesize Bcl-2 production. Papillomavirus, on the other hand, inhibits the activity of p53, a strong inducer of apoptosis (15).

2. Classification of Virus-Induced Apoptosis

Apoptosis is a type of defense mechanism that infected cells develop against viral agents. In the course of evolution, viruses have developed a number of ways to counteract the premature death by apoptosis of infected cells to prevent their own replication. The strategies used by viruses to evade apoptosis of the host cell are classified into three types (Table 1) (16).

Table 1. Strategies used by viruses to evade apoptotic cell death.

Strategies	**Examples**
Completion of the formation of the next generation of viruses by rapid replication before apoptosis is induced	VSV, NDV, Influenza virus
Antiapoptotic genes suppressing apoptosis	Poxviruses, Herpes viruses (HSV, EBV), adenovirus, poliovirus, HIV, Sendai virus, papovavirus
Latent infection; Failure to activate the signaling pathway required for induction of apoptosis in infected cells	Persistently infected viruses

VSV: Vesicular stomatitis virus, **HIV:** Human immunodeficiency virus type 1,

HSV: Herpes simplex virus, **NDV:** Newcastle disease virus

EBV: Epstein-Barr virus

Although little is known about virus-associated apoptosis processes, four different types of relationships have been identified between the time of apoptosis induction and the viral replication cycle in virus-infected cells (Table 2). In three of these four classifications (Type-1, 2, 3), induction of apoptosis occurs in infected cells. While type-1 and 2 require new gene expression in infected cells, type-3 does not. In type-3, viral agents with limited replication ability for host cell apoptosis induce apoptosis in the presence of inhibitors of protein synthesis (cycloheximide) and protein transcription (actinomycin D). The best examples are herpes simplex virus (type-1, type-2) and vesicular stomatitis virus infections (17,11). A similar situation is observed with specific genetic mutations or treatment with ultraviolet radiation. In type-3, apoptosis is thought to be initiated by binding of virions to cell surface receptors or subsequent entry of viruses into the cell (1).

Table 2. Relationships between the time of induction of apoptosis and the viral replication cycle in virus-infected cells (17,11).

Apoptosis	Cells undergoing apoptosis	Need for virus replication	Time of induction	Examples
Type 1	Infected cells	+	Simultaneous with virus replication	Parvovirus, VSV, NDV, MV Influenza virus, Reovirus, TGEV, Sindbis virus, La Crosse virus
Type 2	Infected cells	+	After replication	Mutant VSV
Type 3	Infected cells	-	Before replication	Vacciniavirus Reovirus HSV-1, HSV-2, VSV
Type 4	In both infected and non-infected cells	-	With or without additional stimulation	HIV-1

TGEV: Transmissible gastroenteritis coronavirus

NDV: Newcastle disease virus

HSV: Herpes simplex virus

HIV-1: Human immunodeficiency virus Type 1

MV: Measles virus, **VSV:** Vesicular stomatitis virus

Most RNA animal viruses cause Type-1 apoptosis in infected cells. These viruses cause the host cell to synthesize its own viral proteins while inhibiting the synthesis of macromolecular proteins required by the cell. This defect leads to apoptosis of infected cells, most likely through the intrinsic pathway. In addition, the formation of virus-associated genes in the host cell also occurs. Some of the gene products, such as interferon regulatory factor (IRF-3), can induce apoptosis (18). However, excessive secretion of viral proteins can induce apoptosis of infected cells via the ER-mediated stress pathway, leading to the opening of folds in the ER (19).

In contrast, some viruses can induce apoptosis in the Type-2 pathway without a known trigger and apoptosis does not occur during replication. Apoptosis observed during infection with a mutant vesicular stomatitis virus (VSV) strain carrying M protein is of this type. In cell cultures, only the secretion of M protein caused apoptosis and this protein is considered to be the main cause of apoptosis. On the other hand, another mutant strain of VSV carrying a structurally altered M protein was found to cause apoptosis long after viral replication (20). In this case, apoptosis was interpreted to be the result of nonspecific degeneration in infected cells rather than the direct effect of M protein (1).

Type-4 apoptosis is thought to be caused by interactions between viral particles and cell receptors or by viral proteins released from infected cells. This interaction directly induces apoptosis pathways in cells, regardless of whether the cells are infected or not. This form of apoptosis was first identified in the widespread death of CD4+ T-lymphocytes in HIV1 infection in humans (21,1). However, in other retroviruses, as in AIDS, apoptosis works against host defenses, further increasing the severity of infection (21).

AIDS and FIV (Feline immunodeficiency virus) are diseases characterized by progressive and selective reduction of CD4+ T-lymphocytes. The pathogenesis of these viruses that kill immune cells has not been fully understood. Most T-lymphocyte cells that die during HIV infection have not been found to be infected with the virus and therefore the number of apoptotic T-lymphocyte cells is not associated with the direct effect of the virus (22). Until recently, it was thought that HIV (Human immunodeficiency virus) virus infected CD4+ T-lymphocytes and the number of these cells in the blood decreased as they reached a high mortality rate. However, recent studies do not support this view (23). It has been found that only 1/100.000 of CD4+ T-lymphocytes in human blood are infected with the virus. This raises the question of why the large numbers of uninfected CD4+ T-lymphocytes die. Although the mechanism

is still not fully understood, research suggests that apoptosis may play a role in this event. All CD4+ T-lymphocytes (infected and uninfected) express Fas receptors. Expression of the HIV gene Nef causes HIV-infected cells to express large amounts of the Fas Ligand molecule on the cell surface. FasLs of infected CD4+ T-lymphocytes combine with Fas receptors of uninfected cells to induce apoptosis of healthy CD4+ T-lymphocytes. As a result, apoptosis does not work to the benefit of the organism here, but on the contrary, it works to its detriment and adversely affects the course of the disease (24).

Another disease in which apoptosis does not work as a defense mechanism for the benefit of the organism is that HSV-1 causes hepatitis by causing severe apoptosis in hepatocytes (25). In addition, in most of the diseases in the paramyxovirus family (bovine plague, small ruminant plague, distemper and measles), it is observed that the immune system collapses with the induction of apoptosis with necrosis, especially in lymphoid tissues and peripheral blood lymphocytes and macrophages. A similar situation is also noted in parvovirus infections (26).

3. Strategies of the Viruses to Evade Apoptosis

3.1. Evading Apoptosis with Rapid Replication

The fact that most RNA viruses can continue to develop under apoptotic conditions puts the role of apoptosis as a host defense mechanism in a controversial position. To demonstrate the importance of apoptosis in RNA virus infections, viral replication titer and apoptotic process were compared in HeLa cell cultures infected with vesicular stomatitis virus (27). VSV virus triggered apoptosis in most of the infected cells within the first 5 hours of replication. While viral replication and apoptosis kinetics were parallel in the first 5 hours in which new generation viruses were formed, in 8-10 hours, viral replication kinetics drew an increasing graph compared to apoptosis. Thus, although apoptosis was induced in VSV infection, the virus escaped apoptosis by increasing its replication rate. Similar results were recorded in studies conducted with influenza virus (28). While apoptosis was suppressive for the first generation of some RNA viruses, subsequent generations of viruses have discovered a way of rapid replication to avoid it (1).

3.2. Evading Apoptosis with Antiapoptotic Genes

DNA viruses take a different route to avoid apoptosis than RNA viruses. Unlike the RNA viruses mentioned above, most DNA viruses (poxviruses,

herpesviruses and adenoviruses) do not cause apoptosis of the cells they infect. The anti-apoptotic genes developed by this group of viruses against apoptosis of the host cell are shown as the reason for this (29). Infected cells present viral peptides with major histocompatibility class I (MHC-I) surface receptors to ensure recognition and killing of infectious agents by cytotoxic T-lymphocytes. Cytotoxic T-lymphocytes also activate proteases using perforin or activation of Fas receptors on the target cell surface to induce apoptosis in target cells. Many viruses are able to disrupt the normal pattern of apoptosis in infected cells in order to subvert host defenses. To achieve this goal, viruses inhibit apoptosis by blocking one of the two main control points leading to apoptosis with antiapoptotic genes encoding important inhibitor proteins (30). Cowpox virus antiapoptotic gene crmA specifically blocks caspase-1 and caspase-8 and prevents Fas/FasL-mediated apoptotic cell death (31-34). Other antiapoptotic viral genes encode a protein with structural and functional similarity to Bcl-2. For example, BHRF-1 of Epstein-Barr virus, LMW5-HL of African swine fever virus and EIB antiapoptotic genes of adenovirus encode Bcl-2-like proteins (35,36). Some viral gene products, such as LMP-1 from Epstein-Barr virus, can regulate the Bcl-2 protein in order for the virus to persist in the host cell (37). How the p35 gene, an inhibitor of apoptosis in baculovirus, prevents the death of infected cells has not yet been elucidated. Interestingly, the other baculovirus gene (inhibitor of apoptosis gene, IAP) is analogous to a gene associated with the pathogenesis of a recessive neurodegenerative disorder observed in children (38). Interestingly, the other baculovirus gene (inhibitor of apoptosis gene, IAP) is analogous to a gene associated with the pathogenesis of a recessive neurodegenerative disorder observed in children (38). Apoptosis is not observed during natural infection with herpes simplex virus-1 (HSV-1). However, in invitro studies, apoptosis of HSV-1 infected cells in the presence of cycloheximide is remarkable. In a study conducted to demonstrate the antiapoptotic activity of HSV, both HSV-1 infected and uninfected HEp-2 cells were tested in cultures containing 1 M sorbitol; and it was found that sorbitol-induced apoptosis decreased over time in HSV-1 infected cells, but apoptosis was maintained in uninfected cells. This was considered as the biggest evidence that HSV-1 genome contains an antiapoptotic gene (10). Later, the HSV-1 antiapoptotic gene was described in detail and named as the Us3 protein kinase gene (39,40). This gene prevents apoptosis by preventing the activation of proapoptotic Bad from the Bcl-2 protein family (41). However, while HSV-2 carrying Us3 gene should prevent apoptosis in infected cells, apoptosis was noted to occur in the

presence of cycloheximide. In HSV-1 virus, several other apoptosis suppressor genes with a function similar to Us3 were found (42,43). From this result, it was concluded that viruses need multiple antiapoptotic genes to resist apoptosis of infected cells. It is thought that only one antiapoptotic gene may not have much effect. Likewise, the fact that most DNA viruses contain more than one gene to stop apoptosis, an important defense mechanism of the cell against viral infection, supports this idea (1).

RNA Viruses with Antiapoptotic Activity

It was mentioned above that the only strategic pathway that RNA viruses take against the apoptotic response of the host cell is rapid replication. In this way, a new generation of virus is formed at a sufficient titer before infected cells undergo apoptosis (11,16). In addition, the fact that many of these viruses reproduce in the cytoplasm, partially independent of the host cell nucleus, further increases the rate of replication. It is understood that most RNA viruses have avoided apoptosis in the evolutionary process by choosing to replicate rapidly rather than trying to possess antiapoptotic genes. On the other hand, it was noted that HIV-1, a member of the retrovirus family, and one of the few RNA viruses that inhibit apoptosis, suppressed apoptosis in HEp-2 cells even in the presence of the antiapoptotic gene sorbitol (44). When HIV-1 virus enters the host cell, it requires a long period of time for reverse transcription, integration into the host cell DNA and expression of the virus genome, which results in a slow replication rate. For these reasons, it is considered natural that HIV-1 has developed antiapoptotic genes, as in DNA viruses, to avoid the destructive effect of apoptosis (1).

Poliovirus with a small RNA genome, which is a member of the picornavirus family, has antiapoptotic genes despite its ability to multiply very rapidly (45). Antiapoptotic activity of polioviruses was found in cells treated with sorbitol during infection. Polioviruses transiently suppress both apoptosis and necrosis in infected cells. Polioviruses quickly and efficiently cause cytopathic effects (CPE), a sign of necrosis in infected cells. The virus must suppress both necrotic and apoptotic cell death in order to provide sufficient time for its own replication before CPE begins (12).

Unlike poliovirus, Sendai virus causes persistent infection and replicates very slowly. While poliovirus completes a single cycle of viral replication in HEp-2 cells in 8 hours, Sendai virus completes this cycle in approximately 40 hours. Although the field strain of Sendai virus does not induce apoptosis in

HEp-2 cells, a mutant Sendai virus lacking C proteins causes severe apoptosis with loss of viral replication. These results indicate that the viral C protein suppresses apoptosis in HEp-2 cells, allowing increased viral replication (5).

3.3. Evading Apoptosis with Persistent Infection

In order for persistent infection or very slow viral infection to occur, the virus must not interfere with the critical functions of the host cell. Another purpose of antiapoptotic genes is to prepare the ground for the development of persistent infection (8). In addition, in infections caused by viruses that do not have these genes, persistent infection may occur if apoptosis of host cells is not triggered. This type of infections can also be defined as "cryptic infection" (1).

Studies show that Sendai virus replicates through persistent infection (1). In natural cases, Sendai virus frequently causes persistent infection and leads to very few cells death in tissues to which it has affinity. The fact that Sendai virus rarely grows in cell cultures by forming CPE confirms this. On the other hand, Newcastle disease virus, another member of the paramyxovirus family, is well known to activate apoptosis in infected cells in both in vivo and in vitro studies (5). This situation is not different for other members of the group such as bovine plague, small ruminant plague and distemper viruses (1). The latent infection hypothesis is tried to be explained by studies conducted with Sendai virus. According to these studies, it is thought that the antiapoptotic C protein released by the virus fulfills more than one function in infected cells. Compared to other antiapoptotic genes and antiapoptotic elements, the targets of C protein are thought to be different. The functions of C protein can be listed as suppression of the antiviral functions of IFN, regulation of the synthesis of viral RNA and formation of the virion. It is likely that C protein not only suppresses apoptosis but also predisposes to persistent infection. Since this information is not sufficient, "persistent infection" is still a hypothesis in the pathogenesis of viral diseases (1).

4. Effects of Inflammatory Cytokines on Apoptosis in Viral Diseases

To neutralize the infection, the host initiates an inflammatory response. The main components of this response are interferons, cytotoxic T-lymphocytes, antibody synthesizing B-lymphocytes and complement. These components work in concert, enhancing each other's action to destroy the virus in the host cell. In this process, the inflammatory response causes many clinical symptoms

and lesions. Interferons (α, β and γ) are produced by virus-infected cells. These cytokines work to stop the next generation of virus replication in infected and neighboring cells. Interferons also increase antigen expression in infected cells, making them more easily recognized by cytotoxic T-lymphocytes. Cytotoxic T-lymphocytes initiate the destruction of infected cells by releasing perforins that open pores in the plasma membrane. Subsequently, cytotoxic T-lymphocytes discharge granzymes that degrade cell components and activate caspases into the infected cell, stimulating apoptosis of the host cell (46).

Some viruses (e.g., cytomegalovirus, bovine herpes virus I, adenoviruses) reduce the expression of MHC-I antigens on the surface of the infected cell. Thus, cytotoxic T-lymphocytes are unable to detect viral antigens that do not form complexes with MHC-I. As a result, apoptosis may not be induced in infected cells and viruses may survive. In contrast, cells lacking or deficient in MHC-I on their surface are recognized by natural killer cells that cause cell death using similar pathways as cytotoxic T-lymphocytes (30).

Specific antibodies are secreted to neutralize the new generation of viruses that survive the destructive effect of apoptosis and leave the cell. Circulating antigen-antibody complexes activate the complement system. This leads to stimulation of inflammation, effective neutralization of the virus and destruction of infected cells by necrosis (30).

In natural viral infections, infected cells, macrophages and lymphocytes secrete various cytokines including interferon (IFN), TNF (tumor necrosis factor), interleukin-1 and 6 to regulate inflammation (5). Among these inflammatory cytokines, IL-1 and IL-6 are responsible for acute phase reactions in the organism, but they are not directly related with apoptosis. Some viruses have various cytokine receptors (e.g. vaccinia virus). Cytokines secreted by immune cells may not be sufficiently effective in the organization of the immune response by binding the virus. In particular, they inhibit the activation of cytotoxic T lymphocytes and macrophages and suppress the induction of apoptosis. For these reasons, some viruses are thought to form many serotypes as an escape mechanism from apoptosis (30).

On the other hand, IFN α-β-γ distinctly inhibits direct viral development and consequently apoptosis is suppressed (5). While some scientists have emphasized that interferon α and β cytokines may act as the main mediators of virus-associated apoptosis, no results have been obtained to confirm this view in VSV and HSV1 infections (5). Although interferons help the formation of apoptosis in some viral infections, this could not be confirmed

for each viral infection. Therefore, the efficacy of interferons on apoptosis is still debated (8).

TNF is a typical example of inflammatory cytokines and its antiviral effect against viral replication is at a lower level compared to IFN (47, 48). However, it is a cytokine with strong antiviral activity because it inhibits viral development by inducing apoptosis in infected cells. Studies with VSV on the rate of viral development and induction of apoptosis have revealed that TNF significantly reduces VSV development by inducing apoptosis of infected cells. During infection, apoptosis accelerated up to 1 hour faster in TNF-treated cells compared to control cells (11). Although VSV uses the rapid replication pathway to overcome apoptosis, TNF induced apoptosis of infected cells at an earlier period, which significantly inhibited viral replication (27). In fact, similar results were also found in influenza virus-infected cell cultures (5). Against the apoptotic effect of TNF, some poxviruses encode homologs of TNF-R1 receptors and neutralize all actions of TNF and cytotoxic T-lymphocytes (47). Adenoviruses prevent apoptosis by causing loss of TNF-R1 and Fas receptors through E3-10 and E3-14 proteins they secrete. In conclusion, these studies reveal that TNF makes important contributions to the host defense mechanism during infection through apoptosis (1).

The role of virus-associated apoptosis in the host defense mechanism is to suppress viral replication by providing recognition and digestion of infected cells by macrophages. However, it has been reported that apoptosis is not very effective in the development of virus-specific immunity compared to necrosis (16). In fact, studies on immunity in viral infections show that apoptosis may have a negative effect on the immune response (49, 50). Autoimmune diseases should be considered if inflammatory reactions are triggered only by phagocytosis of apoptotic cells. Sequential immunologic reactions require antigen presentation by macrophages and various cytokines. Apoptotic cells, unlike necrotic cells, do not cause cytokine production. However, it has been noted that in some cases apoptotic cells may provide cytokines necessary for the initiation of an inflammatory reaction and may partially contribute to the development of virus-specific immunity. In viral infections, the severity of the immune response is determined by the degree of necrosis rather than apoptosis (1).

The importance of virus-associated apoptosis depends on both the type of virus and the type of cell. In in vitro studies, virus-associated apoptosis contributes significantly to the host defense mechanism by preventing viral replication and release in infected cells. However, in infections such as FIV and

HIV, apoptosis can trigger the onset of the disease by paralyzing the immune system due to its destructive effect on immune system cells. Considering the suppressive role of apoptosis in the immune system, the presence of necrotic cells instead of apoptosis is intriguing. This suggests that necrosis plays an important biological role in the induction of virus-specific immunity, although strong necrosis at the site of infection initiates extensive inflammatory reaction. A balance of apoptosis and necrosis is essential for infected animals. Further studies in in vivo host-virus interactions should clarify the importance of apoptosis and necrosis in the early genetic response to viral infection and the need for these reactions (1).

5. Conclusion

The death of virus-infected cells through apoptosis, which has important activities in determining how biological mechanisms will result in many physiological and pathological processes, becomes a powerful defense option in innate immunity (19, 8). The death of infected host cells results in the blockage of viral replication and thus inadequate viral load in tissues (19, 8). Prevention of infection, especially before adaptive immunity is formed, represents an important advantage for the organism. However, as a result of the interactions between the host cell and the pathogenic agent, viruses have put forward various strategies to stop or interrupt the apoptotic cell death mechanism in order to overcome this defense developed in the host organism (16, 5). With these developed ways, viruses struggle to exist in nature by forming new generations. The data and inferences obtained from the studies show that there is a very delicate balance between the host immune system and disease-causing agents in the formation of diseases through the apoptosis mechanism and that any shift of this balance in any direction will have critical consequences.

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CHAPTER VI

PCR AND ITS ROLE IN BİOCHEMİSTRY

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1. Introduction

The technique used to rapidly create millions of copies of a target DNA (deoxyribonucleic acid) region is called Polymerase Chain Reaction (PCR) (1). PCR has become the standard method in many molecular biology laboratories where nucleic acid research is conducted. Its speed, simplicity, and cost-effectiveness have made it the most widely used and almost indispensable technique from the past to the present.

PCR technology allows for work with even small amounts of genetic material. Its ability to work across a wide range of organisms, from the smallest nanometer-sized organisms to the most advanced life forms, has made PCR one of the most valuable techniques. PCR has been applied and continues to be applied in various fields, including molecular biology and microbiology techniques, molecular cloning and recombinant DNA technology, detection and quantification of changes in gene expression, determination of genes in organisms such as bacteria and viruses, determination of unknown sequences, diagnosis of various diseases, investigation of mutations, identification of genetic markers for cancer, evolutionary biology, immunology, forensic science,

and identity identification studies (2,3). PCR has made significant contributions to illuminating and solving problems in all these areas of use.

2. Brief History of the Discovery of PCR

Since the discovery of DNA, new breakthroughs have rapidly gained momentum, paving the way for the development of molecular biology and genomics. The discovery of the PCR technique dates back to the early 1980s. PCR was developed as a result of significant research conducted by American biochemist Kary Mullis at Cetus Corporation in 1985. In 1993, Kary Mullis was awarded the Nobel Prize in Chemistry for this groundbreaking discovery (4). Initially, DNA polymerase enzyme isolated from *E. coli* bacteria in 1956 was used for the original PCR. However, it was reported that this enzyme lost its activity irreversibly at high temperatures, necessitating the addition of fresh enzyme in each PCR cycle. In 1976, the Taq polymerase enzyme isolated from the thermophilic bacterium *Thermus aquaticus* offered a solution to this problem. Taq polymerase, which could maintain its activation at high temperatures, eliminated the need for continuous addition of polymerase enzyme after each cycle (5). Subsequently, water baths used for PCR cycles were replaced by the invention of thermal cyclers. This allowed for easy automation of the device and faster completion of experiments. Even today, issues such as specificity, sensitivity, reaction mixture composition, cycle numbers, and contamination remain important for each study and continue to be optimized.

PCR has been a cornerstone invention in the completion of the Human Genome Project (2). Over time, PCR technology, with contributions from numerous scientists, has seen significant advancements, especially in the development of PCR variants such as Reverse Transcription PCR, Real-Time PCR, and Digital PCR, which have revolutionized the field.

Certainly, the PCR technique has become widely known due to the global pandemic that has deeply impacted the world. In December 2019, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the pathogen causing the coronavirus disease (COVID-19), spread worldwide from Wuhan, China, and was declared a pandemic by the World Health Organization (6). PCR played a critical role in the diagnosis and identification of the rapidly spreading disease from its early cases. RT-PCR provided a rapid and reliable method for detecting SARS-CoV-2 infection (7).

3. Classic PCR Strategy

PCR has a simple working principle and is reminiscent of in vivo replication, which plays a role in the transfer of genetic information. The replication principle is based on the separation of DNA strands and the reformation of each strand into a double helix under isothermal conditions, without the need for a thermal cycler. DNA polymerase enzyme, in collaboration with various auxiliary proteins, makes DNA synthesis possible. Understanding the mechanism of action of these proteins has enabled in vitro isothermal DNA amplification techniques like PCR (8). With the PCR method, millions of copies of a specific region of DNA can be exponentially amplified within a single PCR tube. The final product generated after each amplification cycle is called the amplicon. The basic requirements for a PCR reaction are template DNA, DNA polymerase enzyme, deoxyribonucleotides (dNTPs), and primers.

The basic stages of the PCR method are as follows (3,9);

Denaturation: This constitutes the first stage of PCR. It involves the conversion of double-stranded DNA into a single-stranded state due to the breaking of hydrogen bonds between the purine (adenine, guanine) and pyrimidine (cytosine, thymine) bases at high temperatures (94-96°C). This disrupts the double-helix structure, creating suitable regions for the binding of primers in the next stage.

Annealing: This process occurs when the reaction temperature is lowered (50-65°C), allowing forward and reverse primers to bind to their complementary regions on the single-stranded template DNA. The temperature required for primer binding is called the annealing temperature (Tm). The duration of this stage can vary depending on the length of the DNA to be copied.

Extension: This stage involves the extension of primers bound to DNA through the action of Taq DNA polymerase enzyme. Since the enzyme's optimal working temperature is 72°C, primer extension takes place at this temperature. The enzyme adds free nucleotides in the 5'-3' direction to the 3'OH ends of the primers, allowing the DNA to replicate into double-stranded copies. The addition of nucleotides reestablishes hydrogen bonds between the bases, recreating the helical structure.

As a result, the amount of DNA doubles with each cycle. Each newly synthesized DNA strand consists of one new and one old strand and is an exact replica of the original molecule (Figure 1). The amount of product generated increases proportionally with the number of cycles. DNA fragments obtained

from PCR can be observed using agarose or polyacrylamide gels under UV light.

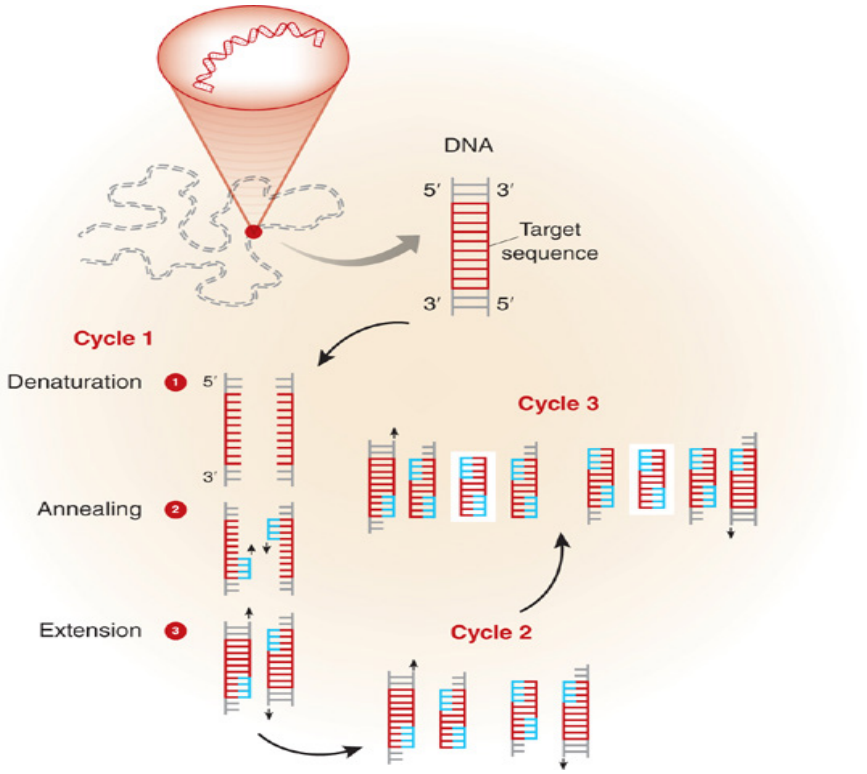

Figure 1. Stages of the PCR Principle (10).

4. Advantages and Disadvantages (11,12);

Advantages of PCR:

- Diagnosis of genetic diseases, cancer, and various infectious diseases, as well as epidemiological studies.
- Detection of microorganisms that are difficult to culture and identify.
- Differentiation between closely related species.
- High specificity for various analyses.
- Despite its complex parameters, PCR is sensitive and precise.
- Speed in detection and diagnosis for experiments that would otherwise take a long time.
- Facilitation of subsequent studies such as cloning and sequence analysis.

Disadvantages of PCR:

- High risk of cross-contamination.
- Sensitivity and specificity issues leading to false-positive and false-negative results.
- High cost due to the need for specialized equipment, consumables, chemicals, and reagents.
- Requirement for well-equipped laboratory facilities.
- Need for PCR optimization depending on the components used and environmental conditions (temperature, humidity, air quality, etc.).
- Dependence on experienced and qualified laboratory personnel.

5. The Basic Components of Polymerase Chain Reaction (PCR)

In the PCR technique, the fundamental components used are template DNA, thermostable DNA polymerase enzymes, primers, deoxyribonucleotides (dNTPs), buffer solution, and magnesium ions (Mg^{+2}).

Thermostable DNA Polymerases: Various DNA polymerases with different characteristics are available for synthesizing large DNA products, improving reaction efficiency, and having unique properties. The choice of which enzyme to use depends on the purpose of the experiment. Typically, for routine PCR, the thermophilic eukaryote Taq DNA polymerase enzyme, isolated from the bacterium *Thermus aquaticus*, is preferred (9). The discovery of Taq polymerase in 1976 in Yellowstone National Park was groundbreaking for PCR because it exhibits activity at high temperatures (13). It plays a role in primer annealing and extension of the DNA strand. While Taq polymerase synthesizes by adding dNTPs to the template DNA in the 5'-3' direction, it lacks 3'-5' exonuclease activity and "proofreading" activity. Taq polymerase carries out the extension step of synthesis at 72°C. During the denaturation step (94°C) repeated during the PCR cycle, Taq polymerase can remain stable at 72°C (14). However, at lower temperatures, a decrease in the extent of strand extension can occur. Commercially available thermostable DNA polymerases have become an indispensable part of the PCR technique due to the adaptability of PCR automation.

Primers: Primers are short, single-stranded artificial DNA fragments typically 15-30 base pairs long. They are shorter than the nucleotide sequence and are used to amplify a specific region of DNA. The determination of forward

and reverse primers defines the DNA segment to be amplified. The forward primer specifies the start of the target DNA region, while the reverse primer specifies the end, and they hybridize to complementary regions of the DNA during synthesis (15). Primers bind to the 3' ends of opposite DNA strands at temperatures ranging from 37-55°C in the 5'-3' direction. The length and base content of the primer affect the annealing temperature (16). Specificity decreases when the temperature is not suitable. Therefore, determining the temperature range for primer binding is crucial. Additionally, it's important that primers are not complementary to each other to prevent the formation of secondary structures like primer-dimers (17). When designing or purchasing primers, it's better to avoid selecting primers rich in AT and GC content, as this can yield better results.

Template DNA: The double-stranded genetic material that contains the DNA region to be amplified serves as the template in PCR. Genomic DNA, yeast, bacteria, viruses, various genes, plasmids, and phage DNA can all be used as templates in PCR. The initial DNA quantity used in PCR varies depending on the organism. DNA isolated from body fluids (blood, saliva, etc.), organs, and tissues should be isolated according to protocols and stored under appropriate conditions. The obtained DNA should be as pure as possible because chemicals used during isolation such as phenol, Proteinase K, and EDTA can inhibit Taq Polymerase enzyme activation (16). The target chain doesn't always have to be DNA. In PCR where RNA is used as a template, RNA is first converted into complementary DNA (cDNA) in a reaction catalyzed by the reverse transcriptase enzyme and can then be used as a template. This procedure is quite common in cloning techniques. As the length and molecular weight of the target DNA increase, the success rate of the copying process may decrease. With PCR amplification, it only takes a few hours to obtain approximately 100 billion copies from a single DNA molecule.

Magnesium (Mg^{+2}) Ions: Enzymes require cofactors to become active, and DNA polymerase enzymes use Mg^{+2} divalent cations as cofactors for activation. Magnesium ions bind to dNTPs, forming a substrate for Taq polymerase enzyme. However, for free Mg^{+2} ions to function as cofactors in PCR, the concentration of Mg^{+2} ions needs to be higher than that of dNTPs. The concentration of Mg^{+2} ions is crucial for the binding between primers and template DNA. Low concentrations of Mg^{+2} ions result in weak primer-template DNA binding, while high concentrations can lead to non-specific binding (9,16). Therefore, the Mg^{+2} ion concentration should be added to the

PCR mixture at an optimal level, taking into account the concentrations of other PCR components.

Deoxyribonucleotides (dNTPs): dNTPs are composed of DNA nucleotide bases, including dATP, dTTP, dCTP, and dGTP. In a standard PCR, the concentrations of dNTPs are equal. They are commercially available in high-purity solutions either individually or as a mixture of all four. The amount of dNTPs and the conditions of use are important factors to consider for PCR sensitivity. Since dNTP stock solutions are sensitive to freezing and thawing, they should be stored in small aliquots (2-5 µl) at -20°C in 10 mM Tris (pH 8.0) (9).

Buffer: Electrolytes and coenzymes required for enzyme activation are provided by buffer solutions. The composition of the buffer solution is determined based on the type and properties of the enzyme used. It typically contains divalent cations ($MgCl_2$), monovalent cations (KCl), Tris-Cl (pH 8.3-8.8), and $(NH)_2SO_4$ (18,1).Formun Üstü

6. Types of PCR

Real-Time PCR: Real-Time PCR is a popular method that allows for the monitoring of amplification products, which can be DNA or mRNA molecules, during analysis. This technique uses fluorescent probes and dyes, making it possible to visualize the quantity of amplified products in real-time (19). In Real-Time PCR, the quantity of the product is directly proportional to the emitted fluorescent light. Because of these fundamental features, it can be referred to in different ways, such as “kinetic PCR,” “homogeneous PCR,” or “quantitative real-time PCR.” There are four main strategies for detecting PCR amplification products: fluorescent dyes that change their fluorescence behavior upon binding to double-stranded DNA (dsDNA), hydrolysis probes, molecular beacon probes, and hybridization probes made up of fluorescently labeled oligonucleotides. Sybr Green and TaqMan probes are the most commonly used fluorescent molecular systems (20). Sybr Green is a dye that emits fluorescence only when bound to double-stranded DNA. Every time the target DNA becomes double-stranded during amplification, it results in an increase in the amount of dye binding and emitted fluorescent light. In the TaqMan system, the probe contains a reporter fluorochrome (6-carboxyfluorescein = 6-FAM) at the 5’ end and a quencher fluorochrome (6-carboxytetramethyl-rhodamine = TAMRA) at the 3’ end. The probe hybridizes to a region between the binding sites of the

primers on the target molecule, which has been unwound into a single strand. As long as the probe remains bound to the target molecule, the signal from the reporter at the 5' end is quenched by the quencher fluorochrome at the 3' end. However, during chain extension, the Taq DNA polymerase enzyme, using its 5'→3' nuclease activity, starts to degrade the probe from the 5' end, releasing the reporter fluorochrome, which can then generate a signal (20). This way, the detection of the amplifying product can be determined simultaneously with the increasing signal (21).

Real-Time PCR finds widespread use in gene expression analysis, single nucleotide polymorphism (SNP) and chromosomal mutation analysis, cancer research, disease diagnosis, and food analysis. It allows for rapid and reliable results by minimizing the need for a large number of tissue samples or cell quantities required in molecular biology and genetic research. Real-Time PCR offers advantages over traditional PCR, including high specificity for the target gene, rapid results, the use of fluorescent dyes, no need for gel electrophoresis after PCR, and the ability to obtain quantitative results (22).

Digital PCR (dPCR): The concept of Digital PCR was introduced in 1992 by Sykes and colleagues (23). The workflow of Digital PCR is similar to Real-Time PCR in terms of using fluorescent molecules. It is considered a new technique for the precise measurement of DNA copy numbers and allows for the amplification of a large number of PCR reactions using very small sample amounts. In this technique, there are numerous small partitions or chambers where individual PCR reactions take place. The reliability and repeatability are very high. Compared to Real-Time PCR, dPCR offers higher sensitivity and specificity (24,25). However, it is more costly than other technologies. It is a method that enables the detection of even a single allele, making it particularly useful in cancer research for detecting rare mutations (26) and viral infections. In recent years, it has gained popularity and become a highly practical and applicable technology.

Reverse Transcription PCR (RT-PCR): RT-PCR is based on the conversion of RNA molecules isolated from cells into complementary DNA (cDNA) using the Reverse Transcriptase enzyme, which makes it possible to use RNA in PCR. The discovery of these enzymes has greatly increased the importance of RNA cloning, sequencing, and characterization in molecular biology. It is the most common, fast, and sensitive method used in gene expression analysis. Additionally, the products obtained from RT-PCR in gene cloning studies can be used as cloning vectors to create gene libraries.

Important considerations in this method include RNA isolation steps, working environment, RNA loss due to RNase activation, and the need to obtain RNA of high purity (27).

Nested PCR: Nested PCR is a technique that reduces non-specific amplification in DNA regions. It provides high specificity and sensitivity by preventing the formation of non-specific products. The increased sensitivity is due to a high number of total cycles, and the increased specificity results from the second primer set binding to sequences produced by the first round. The main difference from standard PCR is the presence of two sets of primers and a two-step PCR reaction. Nested PCR reactions are characterized by two consecutive polymerase chain reactions. The first PCR products are used as templates for the second PCR. The DNA segment amplified by the second set of primers is shorter and located within the DNA segment amplified by the first set of primers. Although it is time-consuming and has a high risk of contamination, its main advantages are its specificity and accuracy (28).

Multiplex PCR: Multiplex PCR is a technique that allows the simultaneous amplification of different targets in a single PCR tube. In Multiplex PCR, two or more primer sets designed for the amplification of different targets are included in the same PCR reaction. This technique can save time, effort, and reagents. Moreover, it enables the simultaneous comparison of multiple amplicons (29). Before performing Multiplex PCR, each primer set must be validated for specificity and efficiency in a single reaction. When visualized by gel electrophoresis, the amplicon sizes should be different enough to create distinct bands. Multiplex PCR is commonly used in mutation analysis, pathogen detection (30), disease diagnosis (31) and other applications where multiple targets need to be amplified and analyzed simultaneously.

Allele-Specific PCR: Allele-Specific PCR (ARMS), also known as the refractory mutation system polymerase chain reaction (ARMS-PCR), is a simple and cost-effective technique used to genotype single nucleotide polymorphisms (SNPs) (32). Four primers are used in a single PCR reaction. The principle of the method is based on the 3’ end of the primer having an incompatibility with the template DNA. The use of allele-specific primers with an incompatibility at the 3’ end makes these primers specific to one allele, while being resistant to the other allele. In other words, for DNA polymerase to extend the primer, the 3’ end of the primer must be a perfect match with the template DNA. Otherwise, PCR amplification will not occur. After PCR, the PCR products can be analyzed directly by agarose or polyacrylamide gel

electrophoresis to determine whether an amplicon was produced and if there are any mutations in the DNA (33,34).

Asymmetric PCR: In Asymmetric PCR, only one strand of the target DNA molecule is amplified. Therefore, the primer specific to that strand is present in much higher amounts. This technique involves different primer concentrations and is not equally balanced (12). To optimize Asymmetric PCR, four different parameters are considered: annealing temperature, primer concentrations, template concentrations, and the number of PCR cycles.

Colony PCR: Colony PCR is used to directly determine the presence of DNA sequences in a plasmid without the need for DNA isolation (35). This feature has made Colony PCR the main advantage of this method. Because of its high efficiency, rapid results, and lower cost, Colony PCR is used in genetic engineering, gene cloning techniques, and CRISPR Cas9 studies.

Hot Start PCR: Hot Start PCR is a method that prevents Taq polymerase activity or the inclusion of modified dNTPs until a specific temperature activation step occurs during the reaction. The goal of this PCR technique is to increase sensitivity and specificity by preventing non-specific amplification and primer dimer formation through an extra temperature application (36). Special DNA polymerase compositions, such as antibodies for temperature-sensitive reaction components, and other auxiliary proteins that block DNA polymerase activity at lower temperatures, can be commercially obtained for Hot Start PCR (37).

Touchdown PCR: Touchdown PCR is a technique based on preventing primer binding to non-specific regions of DNA. Therefore, the annealing temperature during PCR is gradually decreased for each cycle set. The aim is to determine the working temperature for primers with an unknown optimal binding temperature, thus preventing non-specific binding. Consequently, specific amplicons are obtained with accurate matching between the primer and template DNA (38,39).

Assembly PCR: Assembly PCR, also known as Polymerase Cycling Assembly (PCA), is based on the joining of short DNA fragments to create large DNA fragments (12). However, because it involves bringing together many small pieces, careful planning and optimization are required for experiments to be successful. It is essential that all fragments are complementary to each other; otherwise, a complete sequence cannot be produced. In addition to DNA polymerase, DNA hybridization is also used to replicate the DNA sequence in a precise order.

7. The Role of PCR in Biochemical Diagnosis

Biochemical diagnosis is an important medical discipline that combines the principles of biochemistry and molecular biology techniques for the diagnosis of diseases, infections, and other health issues. Polymerase Chain Reaction (PCR) has revolutionized this field, enabling rapid, precise, and specific diagnosis of many diseases (40-42).

Diagnosis of Infectious Diseases

PCR plays a critical role in the diagnosis of viral, bacterial, or fungal infections. PCR-based tests, especially for diseases such as HIV, hepatitis, tuberculosis, COVID-19, and others, allow early detection of the disease and prompt treatment. These tests target the genetic material of infectious agents and can detect their presence in a patient's blood, tissue, or body fluids (43), (44).

Diagnosis of Genetic Diseases

PCR is a significant tool in diagnosing genetic diseases. It is particularly useful for identifying carriers of hereditary diseases and confirming the cause of the disease. PCR-based tests that target specific regions of genetic mutations help assess the genetic risk for both the patient and family members (45).

Cancer Diagnosis and Monitoring

PCR is widely used in cancer diagnosis and monitoring. The detection of abnormal changes (mutations) in the DNA of cancer cells can be accomplished through genetic tests performed using PCR. This aids in determining the type of cancer and monitoring the response to treatment (46,47).

Immunological Diagnostics

PCR is used for diagnosing diseases related to the immune system. For example, PCR-based tests have been developed to detect the presence of specific antibodies in the diagnosis of autoimmune diseases (48,49).

Microbial Diagnosis and Antibiotic Sensitivity Tests

PCR is used in the diagnosis of microbial pathogens and antibiotic sensitivity tests. By targeting the DNA of microorganisms, the causative agent

of an infection can be identified, and appropriate antibiotic treatment options can be determined (50,51).

Organ and Tissue Compatibility

In applications such as organ transplantation and other cases requiring tissue compatibility assessment before transplantation, PCR is used to evaluate the genetic compatibility between the recipient and donor (52).

8. Conclusion

PCR, especially in recent times, has become the heart of science. It is an outstanding technique that leads the way for scientific research and holds critical importance in terms of contributions to human health. It is a groundbreaking method that forms the basis of almost all molecular techniques involving nucleic acids. Due to its wide range of applications, it offers significant advantages. Its high sensitivity, specificity, and ability to provide accurate results in a short time allow it to be used in the diagnosis and treatment of diseases. In addition, it has become a fundamental component of biochemical diagnosis. This powerful molecular biology technique plays an important role in medical applications and patient treatment by enabling the rapid and accurate diagnosis of diseases, genetic issues, and infections. Thanks to PCR, early diagnosis of many diseases has become possible, leading to increased treatment success.

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