Clinical trial of the efficiency of three different compositions of acaricidal substances against varoosis in honey bee colonies

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ABSTRACT

This study aimed to evaluate and compare the varroacidal efficacy and mite mortality dynamic during autumn treatment of honey bee colonies in two experimental areas (Boychinovtsi-Northwestern Bulgaria and Zlatiya-Northeastern Bulgaria), treated with three available veterinary medicinal substances. The clinical studies were conducted on the efficiency of the three acaricidal combinations (AC) - one, based on 3.6 mg flumethrin/strip (AC-1) and two contents of essential oils (first one with composition: 5 g thymol plus 2 g peppermint oil/lamellae (AC-2), and the second one with composition: 4 g thymol plus 2 g peppermint oil/ lamellae (AC-3), in the autumn of 2017. We used the product containing coumaphos and an additive with oxalic acid for the control treatment. Methods for establishing the levels of *Varroa destructor* infestation in bees and in brood were used according to OIE Terrestrial Manual. After 35 days of AC-1 exposure, 94.5% and 87.82% efficiency were achieved in the apiaries in Boychinovtsi and Zlatiya, respectively. Efficiencies of the combinations tested (AC-2 and AC-3) for 45 days were detected high only in the Zlatiya apiary (97% and 95%), also 91% and 80% in the Boychinovtsi apiary, respectively. The results of the experiments showed the absence of resistance to the tested substances.

Introduction

Varroosis, a parasitic disease caused by *Varroa destructor* mite, is one of the leading problems in honey bee pathology. *Varroa* mites feed on the hemolymph of pupae and adults, which can result in premature mortality (27). The impacts of Varroa mite infestation can be immediate and profound and very often the mite is a vector for a large number of honey bee viruses (30). *Varroa destructor* prevalence must be constantly monitored and control in the European honey bee *Apis mellifera* so that the colonies can develop normally and produce. The most commonly used treatment schemes involve the application of synthetic acaricides to honey bee colonies. Despite their efficacy, these substances create new problems for beekeeping in Europe, one of which is the emergence of resistant mites (1, 2, 9, 20, 21, 28). The acaricidal efficiency varies with factors such as climatic conditions, treatment period, treatment method, the strength of bee colonies, level of infestation, etc. The moderate use of a given agent is a better tactic than administering a higher dose (22).

According to European requirements (6) for acaricidal **veterinary medicinal products** (VMPs) used against ectoparasites in animals, their efficacy against *Varroa destructor* in bees must be more than 90%. On the other hand, the effectiveness of acaricides is influenced by the rapid adaptation and development of *Varroa* mites resistance in the case of prolonged application of the same products or those containing substances of the same chemical groups. Three different mutations at position 925 of the *V. destructor* voltage-gated sodium channel have been associated with the resistance to these compounds.

González-Cabrera et al. (12), show new evidence for the significant correlation of the mutation with resistance and conclude that it is likely that resistant mites have a reduced fitness. Under current conditions, the demands on the quality and safety of bee products are increasing, which has led to a significant increase in the proportion of organic beekeeping (7, 11, 26, 29). This necessitated the development of new products based organic acids, essential oils and plant extracts.

In some studies, the product with 5 g thymol plus 2 g peppermint oil/ lamellae showed an efficiency of over 90% (15, 16, 17). Strips with flumethrin as an active substance has a proven effectiveness over time 99% (15, 17).

Different methods have been described for the detection of *V. destructor* resistance to fluvalinate used in the laboratory or field (1-4, 21, 34). Milani and Vedova (23), using paraffin capsules in the laboratory, found that mite resistance in northern Italy decreased tenfold after five years of no pyrethroid use. Similar results, in which the susceptibility of resistant mite increased, have been reported by Elzen et al. (10). Gracia-Salinas et al. (13), following the laboratory method of Milani (21) on the sensitivity of 10 populations of *V. destructor* against fluvalinate, reported for the first time resistance of *V. destructor* in areas of northern Spain, where LC50 is 25-50 times higher than this for sensitive mites. The incorrect use of VMP with molecule, that contain pyrethroids has led to the development of resistance in *V. destructor* (1).

In Bulgaria, strips with flumethrin have been used for control of *Varroa destructor* in conventional beekeeping for more than 20 years, and products containing fluvalinate as an active ingredient have been used for a long time. These acaricides are representatives of the same chemical group - the synthetic pyrethroids. This gives us the reason by a clinical trials to verify the previous high efficacy - over 99%, of the flumethrin – strips, and to identify whether any resistance of *V. destructor* has been developed. Early detection of *Varroa* resistance to pyrethroids would sharply reduce losses in beekeeping.

This study aimed to evaluate and compare the varroacidal efficacy and mite mortality dynamic during autumn treatment of honey bee colonies situated at two experimental areas influenced by different environmental conditions, treated with three combinations of acaricidal substances and compared with untreated control of honey bee colonies. And check for possible development of *Varroa destructor* resistance to some of them.

Materials and Methods

The experiments were carried out in the period July - October 2017. The guidelines used were those specified in the ordinance on the requirements for the data contained in the documentation for the issuance of a license for the use of VMP (25), technical guidelines for the evaluation of treatments for control of *Varroa* mites in honey bee colonies (14). Bee colonies from two settlements in the district of Dobrich (Northeastern Bulgaria) and in the district of Montana (Northwestern Bulgaria) were used as follows: apiary in the village of Zlatiya with sixty-two total number of bee colonies; apiary in town of Boychinovtsi with one hundred and eight total number of bee colonies. From each of these apiaries, forty bee colonies were selected. The main selection criteria are the strength of the bee colonies and the amount of the sealed bee worker brood.

I. Methods: 1. Determination of colony development by generally accepted zootechnical methods for measuring strength of bees in kg and brood amount in number of brood cells.

2. Methods for establishing the levels of *Varroa destructor* infestation in bees and in brood were used according to OIE, 2013 (24).

3. Calculate the effectiveness of three acaricidal combinations (AC) using the following formula:

Effectiveness = mites killed by AC x 100

(% Reduction) killed by AC + remaining mites

- Mites killed by AC are all mites collected during exposure to the test product;

- The remaining mites are those that are not killed by the AC and fall off after treatment with the control product.

4. The presented results were processed statistically using the computer program "Statistics".

5. The treated colonies were monitored throughout the test period for normal development, the occurrence of adverse more harmful effects of the test substances on the queen, bees, and brood.

II. Information on the combinations of acaricidal combinatons(AC) used in the trial: - AC (1): strips with composition: 3.6 mg flumethrin/strip

- AC (2): lamellae with composition: 5 g thymol, 2 g peppermint oil and excipients ad 51 g /lamellae

- AC (3): lamellae with composition: 4 g thymol, 2 g peppermint oil and excipients ad 51 g / lamellae

- Control product with composition: coumaphos - 32 mg/ml

- Zootechnical Feed additive for bees with Oxalic acid - composition: plant extracts, organic acids and inverted syrup.

The products were administered according to the manufacturer's instructions.

III. Groups - bee colonies: Colonies in both experimental apiaries were grouped as follows:

E (1) - experimental group - 10 colonies treated with 4 strips of product AC- 1 for 35 days.

E (2) - experimental group - 10 colonies treated with 2 lamellae of product AC-2 for 45 days.

E (3) - experimental group - 10 colonies treated with 2 lamellae of product, AC-3 for 45 days.

K- control group - 10 bee colonies, untreated.

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IV. Apiaries: In the apiary in Zlatiya, twenty of the colonies were settled in ten-frame Langstroth-Root (LR) hives, and twenty – and ten-frame Dadant-Blatt (DB) hives, evenly distributed between the experimental and control groups. In the apiary in Boychinovtsi, experiments were carried out on forty honey bee colonies settled in ten -frame Dadant-Blatt (DB) hives.

V. Stages of the study: - Determination of *Varroa destructor* infestation level of bee colonies (bees and brood) of the experimental and control groups before treatment.

- The infestation level of the bees was determined by taking out a brood comb and approximately 200–250 bees were removed in a container with alcohol. After that the container was stir for 10 minutes. The bees were separated from the mites by means of a sieve with a mesh size of approximately 2–3 mm. Bees and fallen mites were counted. The level of infestation of bees with mites was calculated as a percentage.

- The infestation level of the brood is determined by removing pupae from 100 combs cells with a sealed brood and number of found mites was calculated as a percentage.

The experiments included hives with a level of infestation of 300 to 3000 mites per colony.

- Calculation the strength (in kg) and the amount of sealed brood (in number of cells) of the colony before treatment.

- Rapid test for 4 hours to establish resistance of *Varroa detructor* mite to AC-1 (flumethrin-strips), performed on bees from bee colonies in the experimental group E -1 (AC-1).

- Clinical tests of acaricidal activity against *Varroa destructor* – treatment with the selected substances and combinations. The release of thymol and peppermint oil included in the lamellae does not depend on environmental factors. The active substances are released gradually over 45 days.

- Control treatment with the VMP, containing coumaphos (32 mg/ ml) for bee colonies from experimental group E-1 (AC-1) and with feed additive based on oxalic acid for the bee colonies from the other groups (E-2, E-3, K).

- Monitoring the number of fallen mites in the control and experimental groups.

During the experiment, 530 checklists with mites from each apiary were counted. Counting of mites, fallen into each of the hives included in the groups was provided on the 24th hour, 48th hour, 72nd hour and on the 4th, 8th, 10th, 15th, 20th, 25th, 30th, 35th, 40th and 45th day. For every apiary mite counts were carried out every of these dates during treatment (for E (1), and five bee colonies from K groups -11 reads; for E (2) , E (3) and bee colonies from K groups - 13 reads were recorded for each colony), and 24 hours after control treatment – 2 reads.

On day 35, a control treatment of group E (AC-1) was performed (10 colonies) were treated with coumaphos. At the same time five colonies from the untreated group (K) were treated with oxalic acid. On 45th day the control treatments of groups E (AC-2) and E (AC-3) were performed (10 colonies of each group) and the other five colonies of the control group (K) were treated with oxalic acid. We monitored the number of mites, fallen by the control treatment after 24 hours.

- Calculation the strength (in kg) and the amount of sealed brood (in number of cells) of the colony after treatment.

- Determination of *Varroa destructor* infestation level of bee colonies (bees and brood) of the experimental and control groups after the treatment is completed. The infestation level of bees and brood was calculated in the same way as before treatment.

- Calculation the efficiency of the relevant combinations.

VI. Data analysis: The data obtained were processed variationally and statistically on Descriptive statistic – Normal distribution with software STATISTICA 12, Copyright © Stat Soft Inc. 1984-2014 (StatSoft, 2014). It was used to establish the reliability of the obtained statistical differences with One – Way ANOVA analysis (31).

Results

Apiary in Zlatiya: In the apiary in Zlatiya, the experiment used colonies settled in two types of hives – Dadant-Blatt (DB) and Langstroth Root (LR). The different hive types were distributed almost equally in each group. The strength of the colonies before and after the treatment is given on Table 1.

At the beginning of the experiment, most of the mites were concentrated in the sealed brood (Table 2).

After staying the products in the hives in group E-1 (AC-1) for 35 days, the mite infestation level on bees was 0.51±0.18%. In the experimental groups E-2 (AC-2) and E-3 (AC-3) after 45 days the infestation level on bees decreased to 2.05±0.94% for E (2), and in E (3) slightly increased to 3.09±1.05%. The differences being of low significance /$P \leq 0.05$/. Brood rearing in colonies in all groups was stopped. In the control /K / group, the mite infestation level for bees remained almost the same at 3.38±1.06%. The reliability between the experimental groups E-1 (AC-1) and E-3 (AC-3) was low - $P \leq 0.05$ /Table 2/. The resistance test showed an average of 2.3 mites fallen from the product with flumethrin (AC-1) and 0.6 remaining on the bees in the containers. The test proved a lack of resistance to flumethrin.

The data showed that the most significant number of mites dropped out in the E (2) group under the action of AC-2 which has a higher level of infestation/ -

769.0±210.73 mites, while after the control treatment dropped out - 18.4 ± 4.08 mites or 2.3% of all mites. In the control treatment, in the experimental groups E-1 (AC-1) and E-3 (AC-3) dropped out 7% and 2.7% of all mites, respectively. In the untreated group (K), as a result of control treatment mites that dropped out were about 30%.

The effectiveness of the tested products is presented in Table 3.

The condition of colonies at the end of the trials was normal for the season, according to their initial strength (Table 2). No differences were found between DB and LR hives.

Table 1. Development of bee colonies - apiary Zlatiya.

Group	n	Before treatment				After treatment			
		Strength -kg		Brood -number cells		Strength -kg		Brood -number cells	
		x̄ ±Sx̄	Min/max	x̄ ±Sx̄	Min/max	x̄ ±Sx̄	Min/max	x̄ ±Sx̄	Min/max
E-1 (AC-1)	10	2,18±0,17	1,00/3,00	7150±501,39	5300/10200	2,39±0,18	1,50/3,20	410±129,49	0/1100
E-2 (AC-2)	10	2,31±0,10	2,00/2,80	8110±955,04	4100/13100	2,19±0,17	1,50/3,00	-	-
E-3(AC-3)	10	1,82±0,09	1,50/2,25	7080±459,42	4600/9800	1,83±0,11	1,00/2,20	1700±556,78	600/2400
K-control (untreated)	10	1,82±0,13	1,20/2,60	6420±598,11	4100/10000	2,10±0,11	1,75/2,60	1100±561,25	100/2600
Significance of differences between groups		E-2/E-3** E-2/K**				E-2/E-3*		E-1/E-3**	

The statistical analysis tests the differences between treated groups (E-1 (AC-1), E-2 (AC-2) and E-3 (AC-3)) and control group (ANOVA).
* Indicates P≤0.05 significant level.
** Indicates P≤0.01 significant level, Strength of colony (bees in kg) and (Brood in number of cells) in the beginning and the end of experiment; n (replicates) = 10.

Table 2. Infestation level of brood and bees (%) - apiary Zlatiya.

Group	Before treatment				After treatment			
	IL brood -%		IL bees -%		IL brood - %		IL bees - %	
	x̄ ±Sx̄	Min/max	x̄ ±Sx̄	Min/max	x̄ ±Sx̄	Min/max	x̄ ±Sx̄	Min/max
E-1 (AC-1)	0,67±0,67	0/6,67	1,50±0,46	0/3,90	-	-	0,51±0,18	0/1,45
E-2 (AC-2)	5,33±4,07	0/40	3,31±0,75	0,60/7,90	-	-	2,05±0,94	0/9,90
E-3 (AC-3)	2,00±1,42	0/13,33	1,64±0,72	0/6,90	-	-	3,09±1,05	0/11,40
K-control (untreated)	3,33±2,28	0/20	0,44±0,33	0/2,10	-	-	3,38±1,06	0/11,69
Significance of differences between groups	-	-	E-2/K**	-			E-1/E-3* E-1/K*	-

The infestation level (IL) of brood and bees in the beginning and in the end of experiment.
The statistical reliability between the experimental groups E (1) and E (3) is low - P≤0.05.

Table 3. Efficiency (%) of the used combinations - apiary Zlatiya.

Group	n	Fallen mites from the treatment (number) x̄ ±Sx̄ Min/max	Fallen mites from the control treatment (number) x̄ ±Sx̄ Min/max	All fallen mites (number) x̄ ±Sx̄ Min/max	Efficiency (%) x̄ ±Sx̄ Min/max
E-1 (AC-1)	10	691,3±208,16 60/2337	54,9±10,25 9/94	746,2±210,17 82/2419	87,82±3,10 70,17/99,25
E-2 (AC-2)	10	769,0±210,93 148/2306	18,4±4,08 3/39	787,4±214,33 152/2345	97,26±0,53 93,40/98,98
E-3 (AC-3)	10	693,7±242,98 65/2579	19,2±7,05 1/79	713,9±249,00 74/2658	95,84±1,79 81,48/98,72
K-control (untreated)	10	81,1±19,19 18/211	31,56±8,96 1/78	110,9±25,41 19/232	
Significance of differences between groups		E-1/K** E-2/K** E-3/K*	E-1/E-2** E-1/E-3** E-1/K*	E-2/K** E-3/K*	E-1/E-2** E-1/K* E-2/K*** E-3/K**

Efficiency calculated on the basis of group are means of 10 replicates, n = 10.
Different number of stars indicate significant differences among the groups (E-1, E-2, E-3 and K-control): * P≤0.05; ** P ≤0.01, *** P≤0,001.

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Apiary in Boychinovtsi: The experiments in Boychinovtsi's apiary included colonies with similar pre-treatment strength in all groups - from about 1.85 kg to 1.95 kg of bees. During the treatment period, the colonies were in good condition with about 2 kg of bees (Table 4).

At the beginning of the experiments, mites were distributed on the bees and in the sealed brood in experimental groups (Table 5). The infestation level (in %) of brood and bees in Boychinovtsi is given on the Table 5.

The resistance test showed an average of 9.4 mites dropped from the action of flumethrin-strips and 0.6 remaining on the bees in the containers. The efficiency of the tested products is presented in Table 6.

The established efficiency of flumethrin showed that it has a high acaricidal activity, as for the apiary in Boychinovtsi, it was almost 95%, respectively 94.52±1.16 with a moderate degree of reliability in the differences ($P \leq 0.01$) compared to the untreated control group after staying the strips in the colonies for 35 days.

Table 4. Development of bee colonies - apiary Boychinovtsi.

Group	n	Before treatment				After treatment			
		Strength -kg		Brood -number cells		Strength -kg		Brood -number cells	
		x̄ ±Sx̄	Min/max	x̄ ±Sx̄	Min/max	x̄ ±Sx̄	Min/max	x̄ ±Sx̄	Min/max
E-1(AC-1)	10	1,95±0,05	1,75/2,25	3640±395,87	1900/5300	2,00±0,08	1,50/2,25	0	0
E-2(AC-2)	10	1,90±0,04	1,75/2,00	4510±449,31	2200/6300	1,85±0,05	1,50/2,00	100±100,00	0/1000
E-3(AC-3)	10	1,85±0,04	1,75/2,00	4660±385,34	3200/7200	2,07±0,07	1,75/2,25	160±110,75	0/1000
K-Control	10	1,90±0,04	1,75/2,00	3550±368,56	1800/5700	2,17±0,04	2,00/2,25	100±100,00	0/1000

Strength (bees in kg) and Strength (Brood in number of cells) in the beginning and the end of experiment; n (replicates) = 10.

Table 5. Infestation level of brood and bees (%) - apiary Boychinovtsi.

Group	Before treatment				After treatment				Reliability of start / end differences (bees)
	IL brood-%		IL bees-%		IL brood-%		IL bees-%		
	x̄ ±Sx̄	Min/max	x̄ ±Sx̄	Min/max	x̄ ±Sx̄	Min/max	x̄ ±Sx̄	Min/max	
E-1 (AC-1)	6,67±3,97	0/40	4,32±2,09	0,4/22,6	0	0	0,42±0,21	0/1,9	NS
E-2 (AC-2)	7,33±4,91	0/40	9,63±3,85	0,6/31,5	0	0	12,25±7,44	0/72,4	NS
E-3 (AC-3)	3,34±1,49	0/13,3	5,39±1,85	0/20,6	0	0	10,80±3,88	0,6/42,7	NS
K-Control (untreated)	0	0	3,20±1,54	0/16,6	0	0	17,68±9,93	1,4/102,0	NS
Significance of differences between groups	E-3/K*	-	-	-			E-1/E-3*	-	

The infestation level (IL – the calculated percentage of *Varroa destructor* mite of brood and bees before and after treatment.
Different number of stars indicate significant differences among the groups:* $P \leq 0.05$; NS: Not significant ($P > 0.05$).

Table 6. Efficiency (%) of the used combinations - apiary Boychinovtsi.

Group	n	Fallen mites from the treatment (number) x̄ ±Sx̄ Min/max	Fallen mites from the control treatment (number) x̄ ±Sx̄ Min/max	All fallen mites (number) x̄ ±Sx̄ Min/max	Efficiency (%) x̄ ±Sx̄ Min/max
E-1 (AC-1)	10	1313,9±619,54 277/6651	36,5±6,60 15/88	1350,4±624,70 317/6739	94,52±1,16 87,38/98,86
E-2 (AC-2)	10	643,8±288,78 114/2295	43,7±17,15 5/176	687,5±300,35 128/3099	91,61±1,43 82,97/97,37
E-3 (AC-3)	10	318,8±104,97 34/1046	54,9±15,31 5/138	373,7±116,49 71/1184	80,07±5,24 47,89/93,70
K-Control (untreated)	10	57,4±27,14 10/299	22,9±5,33 0/52	80,3±28,93 22/333	
Significance of the differences between the groups		E-3/K*		E-3/K*	E-1/E-3* E-1/K** E-2/E-3* E-2/K**

Different number of stars indicate significant differences among the groups (E-1, E-2, E-3 and K-control): * $P \leq 0.05$; ** $P \leq 0.01$; Efficiency calculated on the basis of group are means of 10 replicates, n = 10.

The treated groups' observations showed that the substances do not adversely affect on the bees and brood. Self-replacement of the queen was not observed in the experimental and control groups. The development of the colonies at the end of the experiments was normal for the season, according to their initial strength (Table 4).

Discussion and Conclusion

The results obtained from the clinical trials of AC-1, with strips containing flumethrin, show high efficacy against varroosis. After an exposure of 35 days, efficiency is achieved, 94.5% / Min/Max - 87.38 /98.86%/ in Boychinovtsi and 87.82% in Zlatiya, respectively.

In Zlatiya, the lower efficiency of E (AC-1) is due to some technical problems during the experiments. The hives were placed close together, and the control groups were intermixed between the experimental groups, which is why mite re-infestation is possible. In addition, robberies are occurred between some of the colonies in the control group. In several hives of the same group the strips had fallen to the bottom, some of them thrown out by bees.

We believe that due to the high efficiency (over 90%, and in some individual experimental colonies up to 99%), which the flumethrin showed in both apiaries it is a suitable acaricide for the *Varroa* mite prevention and control in the conditions of our country and can meet the needs of beekeeping practice. The lack of harmful effect on bee colonies and the lack of mite resistance make it an excellent substance for controlling varroosis in Bulgaria. Based on the results of the rapid test for resistance and efficacy, we can say that, for the time being, it cannot be claimed that the resistance of *V. destructor* to flumethrin has been established in the test areas. The other tested substances combinations – AC-2 and AC-3 also showed high efficiency. For the apiary in Zlatiya 97% for AC-2 and 95% for AC-3, respectively, and for Boychinovtsi 91% for AC-2 and 80% for AC-3, respectively. Despite the lower thymol content of the AC-3 (with 4 g thymol/lamellae), no highly reliable differences were found in the efficacy of the two combinations in any of the apiaries included in the experiment. Due to the fact that these combinations have no harmful effect on bees, their natural composition, and the lack of residues in honey, they are preferred for use to combat varroosis in organic beekeeping. In addition to acaricidal effects, the application of VMP, containing essentials oils, into hives often also causes antimicrobial effects, which can lead to an overall improvement in the health status of honey bee colonies (5, 8, 19, 33).

It can be concluded that an adequate *V. destructor* mite control must include a few measures, firstly good beekeeping management practice in combination with the appropriate use of authorized acaricidal substances. Different treatment regimes should also be applied with continuous parasitic mite mortality monitoring. Consequently, there is a need to review research that supports a combination of multiple strategies available for *Varroa* control (18). To avoid re-infestation varroacides should be applied after the main honey flow, on all apiaries of the same area, and in all honey bee colonies with mite infestations levels above the economic threshold (32). According to Almecija et al. (1) in the absence of tau-fluvalinate treatment (> 2 years), the susceptible genetic profile is present at 97% of the mites. This seems to imply that *Varroa* mites can regain their sensitivity to tau-fluvalinate quite quickly (after 2 years minimum without tau-fluvalinate treatment for their study. Knowledge of the reversion period for tau-fluvalinate can play a crucial role in the control against the establishment of *Varroa* mite resistance to pyrethroids.

The most used chemical acaricides must be included in rotation programs to decelerate the resistance of *Varroa destructor* mites to multiply used products and reduce the impact of increasing comb wax contamination.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

KG, IZ and DS conceived and planned the experiments. KG and DS carried out the experiments. KG and DS contributed to sample preparation. KG, IZ and DS contributed to the interpretation of the results. IZ analyzed the data. DS took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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