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ORIGINAL ARTICLE

EVALUATION OF ALFAXALONE'S ANTI-INFLAMMATORY AND ANTINOCICEPTIVE EFFECTS IN BROILER CHICKS

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Summary

Background: Progesterone is the source of the steroid general anesthetic Alfaxalone, which became commercially available in the 1970s when combined with alfadolone, another steroid anesthetic.
Objective: the goal of the present research was to discover and assess the sub-anesthetic effects of Alfaxalone: the antinociceptive and anti-inflammatory properties in broiler chicks.
Methods: One hundred and three Ross broiler chicks (80-100 g, 7-8 days old) were used. The electric stimulation test, hot water test (HWT), and formalin test were performed on broiler chicks to assess the antinociceptive and anti-inflammatory properties of Alfaxalone. The up-and-down method that described by Dixon was used to determine the median effective antinociceptive dose.
Results: The median effective antinociceptive doses (ED_{50s}) of Alfaxalone by electric stimulation test and hot water test were 0.94 and 0.65 mg/kg intraperitoneally, respectively. Alfaxalone, at doses of 1, 2, and 4 mg/kg intraperitoneally, induced an antinociceptive effect in the electric stimulation test and HWT as well as antinociceptive and anti-inflammatory effects during the formalin test in the chicks. The antinociceptive effect was displayed 15 min after treatment and the maximum effect was observed 30 min after injection.
Conclusion: The results showed that Alfaxalone had antinociceptive and anti-inflammatory effects, with a stronger antinociceptive effect in the electric stimulation test than in the thermal stimulation test, which was illustrated by shorter antinociceptive time.

Key words: broiler chicks; Alfaxalone; electrical stimulation; hot water test; anti-inflammatory

Introduction

Alfaxalone is a hydrophobic anesthetic neurosteroid molecule and its use has been reported in a wide range of animal species including poultry (1). It is a versatile anesthetic induction agent, largely due to its fast onset of action,

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short duration of action, and multiple routes of administration (intravenous, intramuscular, subcutaneous and intra-osseous) (1). Alfaxalone was utilized in the induction and maintenance of anesthesia in bird species (2–4), dogs (5), cats (6), and humans (7). It was marketed originally in a mixture with alfadone (Saffan), another synthetic neurosteroid, and solubilized in ethoxylated castor oil known also as (Cremophor-EL) which caused allergic reactions (8). The existing formulation (Alfaxan, Jurox Ltd., Crawley, West Sussex RH10 1DD, UK) used 2-hydroxypropyl-b cyclodextrin as a solubilizing material and has not been related to hypersensitivity and allergic reactions (9).

Its mechanism of action was related to its agonist activity on the gamma-aminobutyric acid A ($GABA_A$) receptors producing central nervous system depression and muscle relaxation (10, 11). Lower concentrations of Alfaxalone potentiated the opening of the $GABA_A$ receptor channel, like that formed by benzodiazepine compounds (10). Conversely, Alfaxalone at a higher concentration act as a $GABA_A$ receptor agonist, similarly to barbiturates and propofol (10). So a sub-anesthetic intramuscular dose of Alfaxalone might yield sedative action, the effect is similarly leading to hyperpolarization of the cell, just slightly A different mechanism. Overall, the anesthetic agents have more than one effect on the body, therefore the newer agent must be studied in all laboratory animals to assess their pharmacological effects and safety.

The goal of our research was to assess the antinociception and anti-inflammatory impacts and usefulness of Alfaxalone in the birds, using the broiler chicks as a model thereby providing clinical pharmacological information about the Alfaxalone.

Methods and Materials

Animals

103 one-day-old post-hatch Ross broiler chicks were purchased from a specialized regional hatchery. The birds were accommodated in a chamber with a temperature of 32-35°C, 23 hours of lighting, and 1 hour of dark and wood flakes as floor litter, by providing feed and water *ad libitum*. Chicks were tested at 7-8 days of age.

Drugs and chemicals

The Alfaxalone (10 mg/ml, ALFAXAN, Jurox Pty. Ltd., Rutherford, NSW, Australia) was diluted in distilled water to obtain the accurate concentrations needed for administration intraperitoneally (IP) in a volume of 10 ml/kg body weight (12). Control groups were injected IP with distilled water at the same volume of administration.

Ethical approval

This study was reviewed by the head and members of the Scientific Committee of the Physiology, Biochemistry and Pharmacology Department at the College of Veterinary Medicine/University of Mosul, and the ethical approval was unanimously approved (approval number UM.VET.2021.2).

Experimental animals models of pain

Determination of the median effective doses (ED_{50s}) of Alfaxalone causing antinociception in Broiler chicks: The analgesic ED_{50s} of Alfaxalone were assessed for electrical stimulation and thermal stimulation alone. The first dose of Alfaxalone was 1mg/kg intraperitoneally as mentioned according to the up-and-down method (13). The electric stimulation was done by electro-stimulator apparatus to assess the occurrence of distress calls in chicks as an indicator of nociception sensation by putting the electrodes on the skin under the wings (14). The thermal stimulation was done by water bath apparatus to determine the pain threshold according to withdrawal time, the chicks were grabbed gently in one hand, its left foot was placed under the fingers, and the right foot was left free to move then dipped in water bath. The time spent per second was measured by a stopwatch (15).

The chicks were assessed separately pre and post- 15 minutes of intraperitoneal administration of Alfaxalone, Then, according to the look or lack of antinociception , the doses of the Alfaxalone will be decreased or increased to 0.2 mg/kg, correspondingly according to the initial dose used.

The median effective dose (ED_{50}) of Alfaxalone is based on the table mentioned (13) and uses the following equation:

$$ED_{50} = Xf + Kd$$

Whereas
Xf = the last dose used in the experiment.
K = a tabular value extracted from the table mentioned by Dixon(13) .
d = the amount of constant increase or decrease in the administered dose.

Dose-dependent analgesic effect of Alfaxalone in chicks by electrical stimulator test

32 chicks were randomly allocated into 4 groups of 8 chicks for each group. The chicks were injected with either distilled water (control) or with Alfaxalone at 1, 2 and 4 mg/kg. For each chick, we determined the lowest voltage that produced a nociception reaction at 0, 15, 30, 60 and 120 minutes after the injection. The increment in the electrical voltage in each group was evaluated in a two-way analysis of variance to determine the significant analgesic reaction of the chicks to Alfaxalone.

Dose-dependent analgesic effect of Alfaxalone in chicks by HWT

32 chicks were also randomly assigned into 4 groups of 8 chicks each. Birds in groups were injected with Alfaxalone at 0, 1, 2 and 4 mg/kg. They were each placed in a water bath that was heated to 55±0.5 ºC. Response threshold was defined as the point at which the foot started to leave the hot water. The reaction times of 0, 15, 30, 60, and 120 minutes were recorded. In order to prevent damage to the skin tissue, the cutoff time was chosen at 20 seconds (16).

Antinociception and anti-inflammatory impacts of Alfaxalone in chicks exposed to the formalin test

28 chicks were randomly distributed into 4 groups of 7 birds each. Nociception and inflammatory reactions were provoked in the chicks by injecting 0.05 ml of 0.1% of formalin into the sole region of the right foot (17–20). The chicks were injected with either distilled water or with Alfaxalone at 1, 2 and 4 mg/kg 15 min previously the formalin administration. After the formalin treatment, we documented within 3 minutes the latency to lift the right foot (using a stopwatch) and the frequency of lifting the right foot in reaction to formalin irritation. Furthermore, we detected the anti-inflammatory influence of Alfaxalone by gauging the foot thickness (millimeter) with an electronic digital caliper (Electron-ics Lab, China) before and after 30min of the formalin treatment.

The anti-inflammatory efficacy (%) was determined as follows
% Anti-inflammatory efficacy =

$$\left[\frac{\text{Alteration in foot thickness of control group-alteration in foot thickness of Alfaxalone group}}{\text{Alteration in foot thickness of control group}}\right] \text{x100}$$

Statistical analysis

Parameters were stated as mean + SE (standard error). Statistical analysis was completed by using one and two-way analysis of variance (ANOVA) followed by Dunnett's test. $P<0.05$ were consider statistically significant. The statistical calculations were achieved using the Statistical Package for Social Sciences version 23 (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp).

Results

The ED_{50s} values of Alfaxalone for the induction of antinociceptive in the chicks by electrical and thermal stimulation were 0.94 (Table 1) and 0.65 mg/kg intraperitoneally (Table 2).

Table 1. Median effective dose (ED_{50}) of alfaxalone injected intraperitoneally for induction of analgesia in chicks by using electrical stimulation.

Variables	Results
ED_{50}	0.94 mg/kg
Doses range	1.2-0.8=0.4 mg/kg
Initial dose	1 mg/kg
Last dose	1 mg/kg
+ or - in the dose	0.2 mg/kg
Number of chicks used	(OXXOX) 5
Minimum-maximum voltage that caused pain	Before alfaxalone injection 6-12
	After alfaxalone injection 7-10

X: pain reaction, O: no pain reaction, The ED_{50} were calculated according to (Dixon, 1980)

Table 2. Median effective dose (ED_{50}) of alfaxalone injected intraperitoneally for generation of antinociception in chicks by using thermal stimulation.

Variables	Results
ED_{50}	0.65 mg/kg
Doses range	1-0.6=0.4 mg/kg
first dose	1 mg/kg
Last dose	0.8 mg/kg
+or - in the dose	0.2 mg/kg
Number of chicks used	6(XXOXOX)
Heat temperature of water bath	55-56 °C
Sings of nociception	Foot withdrawal

We calculated the time needed to foot withdrawal from water bath before and after 15 min of alfaxalone injection
X: pain reaction, O: no pain reaction, The ED_{50} were calculated according to (Dixon, 1980)

Dose- dependent antinociceptive effect of Alfaxalone in chicks by electrical stimulation

Alfaxalone at 1, 2 and 4 mg/kg produced an antinociceptive effect at 15 minutes after injection compared to the control group, and this time was the peak time for antinociception. However, the antinociceptive effect expired gradually after the 60 and 120 minutes in chicks had been tested, respectively, thus Alfaxalone had a relatively short-term effect. The group that injected with the highest concentration of Alfaxalone 4 mg/kg produced an antinociceptive effect a quarter of an hour after injection compared to the control group (Table 3) and (Figure 1).

Table 3. Effect of alfaxalone (1, 2 and 4 mg/kg) on electrostimulation in chicks.

Groups	Increase in pain-inducing voltage in the following times				
	0 (baseline)	15 min	30 min	60 min	120 min
Control group (0)	6.07±0.24	6.27±0.71	6.05±0.40	5.78±0.12	5.78±0.24
Alfaxalone 1 mg/kg	6.52±0.52	7.61±0.44	9.78±0.34*	5.62±0.50	5.83±0.17
Alfaxalone 2 mg/kg	6.65±0.24	7.55±0.19	9.52±0.34*	5.05±0.45	6.61±0.13
Alfaxalone 4 mg/kg	6.38± 0.17	8.72±0.78*	10.86±0.63*	5.91±0.45	5.56±0.47

Dara are mean ± Standard error of eight chicks per group.
*P<0.05, as compared to control.

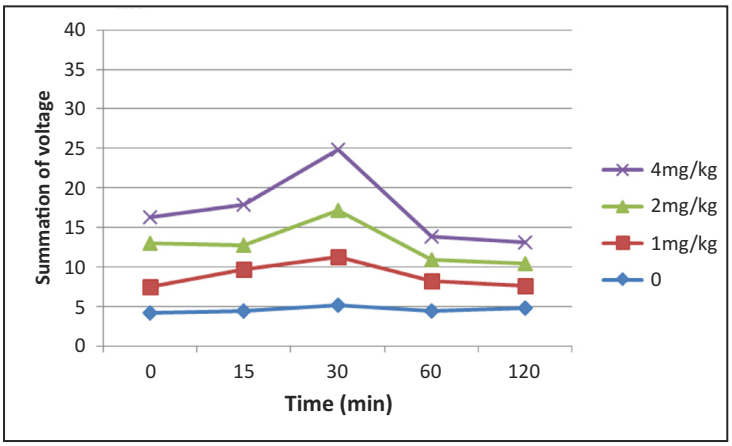

Figure 1. Antinociceptive influence of alfaxalone at 0 (control group), 1, 2 and 4 mg/kg IP. after 15, 30, 60 and 120 minutes of treatment. Struggling and wing flapping was the sign for nociception sensation after increasing voltage of the electrostimulation.

Dose- dependent antinociceptive effect of Alfaxalone in chicks by thermal stimulation

Alfaxalone at 1, 2 and 4 mg/kg created an antinociceptive response at a quarter of an hour after injection compared to the control group, and the peak time for antinociception was after 30 minutes of Alfaxalone injection. However, the analgesic effect expired after the 60 and 120 minutes in chicks had been tested, respectively (Table 4) and (Figure 2).

Table 4. Effect of alfaxalone (1, 2 and 4 mg/kg) on thermal stimulation in chicks.

Groups	The time (s) needed to remove foot from water bath after alfaxalone injection (min)				
	0 (baseline)	15 min	30 min	60 min	120 min
Control group (0)	4.19±0.89	4.43±0.85	5.16±1.21	4.46±1.07	4.81±1.33
Alfaxalone 1 mg/kg	3.29±0.52	5.31±1.49	6.10±0.65	3.76±0.82	2.82±0.49
Alfaxalone 2 mg/kg	2.56±0.36	3.07±0.27	5.86±0.95	2.71±0.42	2.79±0.26
Alfaxalone 4 mg/kg	3.24±0.83	5.10±1.28	7.78±0.83	2.79±0.57	2.72±0.14

Dara are mean ± Standard error of eight chicks per group.

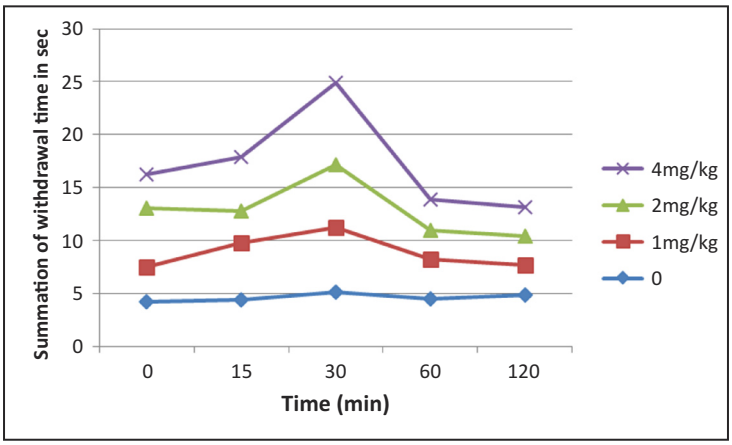

Figure 2. Antinociceptive influence of alfaxalone at 0 (control group), 1, 2 and 4 mg/kg i.p. next 15, 30, 60 and 120 minutes of treatment. Foot withdrawal was the sign for nociception sensation after immersion the foot in the water bath.

Analgesic and anti-inflammatory effects of Alfaxalone in chicks subjected to formalin test.

This experiment showed that Alfaxalone possesses an anti-inflammatory properties and antinociception against formalin injected into the plantar region of the foot of the chick. This was shown by the significantly increased latency to lift the right foot as well as by the significantly decreasing frequency of foot lifting when compared with the control group (Table 5). The anti-inflammatory efficacy of Alfaxalone was shown by the statistically significant reduction in foot thickness compared to the control group, with 8.42%, 38.94%, and 62.10% for Alfaxalone at 1, 2 and 4 mg/kg, respectively, positive responses in the chicks (Table 5).

Table 5. Antinociception and anti-inflammatory effects of alfaxalone injected intraperitoneally in chicks subjected to formalin test.

Alfaxalone mg/kg	Start raising the right foot (seconds)	Rate of recurrence of right foot lifting (counts)	Increment in foot width (millimeter)	Percentage of anti-inflammatory efficacy (%)
0	0.60±0.07	52.71±7.25	0.95±0.13	-
1	0.82±0.06	47.43±4.18	0.87±.022	8.42
2	1.08±0.09*	35.00±3.21*	0.58±0.10	38.94
4	1.52±0.13*ab	26.14±2.21*a	0.36±0.11*a	62.10

Dara are mean ± Standard error of seven chicks per group.
* Statistically significant from the control group, p value < 0.05.
a Statistically significant from the value of the group injected with alfaxalone at 1 mg/kg, p value < 0.05.
b Statistically significant from the value of the group injected with alfaxalone at 2 mg/kg, p value < 0.05.

Discussion

Progesterone derivative Alfaxalone is a relatively high safety margin of neuroactive steroid anesthesia, with minimal cardiovascular, respiratory depression, short initiation and, excitation I in the recovery stage (21). It has a general anesthetic efficacy by acting on the central nervous system in the subtype receptors of gamma aminobutyric acid (GABA) (13). GABA is the most effective CNS inhibitor. Alfaxalone increases GABA effects on the GABAA receptors, which contribute to channels opening into the cells and chloride ion inflows. There are numerous recent documents showing the use of Alfaxalone solubilized in cyclodextrins in mammals, reptiles, amphibians, and fish. Although there are few studies on the effect of the anesthetic Alfaxalone in birds, there are some studies that have been conducted recently (22). In this study, we demonstrate for the first time the median effective antinociceptive dose of Alfaxalone using two different methods of nociception assessment. The ED_{50} for analgesia was 0.94 and 0.65 mg/kg, for electrical and thermal stimulation, respectively. The electrical stimulator test was used to examine and measure the incidence of pain relief in chickens as a qualitative and quantitative method for measuring analgesia (14) and the chick's response to the antinociceptive effect of Alfaxalone was dose dependent. The antinociception peak time was half an hour after the injection of Alfaxalone. However, the effect disappeared after an hour and two after the injection. There are some studies indicating that Alfaxalone has a short duration of action (23–25). We conducted the hot water method (15) using a water bath was used to evaluate the antinociceptive effect of Alfaxalone on the thermal nociception receptors present in the birds (26). To confirm the antinociception action, a formalin test was performed, which consists of two phases to demonstrate the antinociceptive effect (27, 28). Our results showed for the first time that the effect of Alfaxalone has an anti-inflammatory effect, and the reason for this may be its unique composition as Alfaxalone reduced the edema caused by formalin compared to the control group. Some new reports suggested that testosterone which is resembled in structure to Alfaxalone induces an anti-inflammatory effect, The testosterone significantly has an inhibitory effect on the forming of adipose tissues and on the expression of different adipocytokines, including leptin, TNF-α, IL-6, IL-1, whereas the low level of testosterone correlates positively with the increased expression of inflammation markers. Further studies should explore the function of testosterone in the process of development mechanisms and regulation of proinflammation cytokines integrated with weight loss and physical activity (29–31). To confirm the anti-inflammatory efficacy of testosterone, a study conducted by bacterial endotoxin injection into castrated male mice has increased the production of TNFα, which has been abolished by testosterone (32). Several studies

have indicated that Alfaxalone has analgesic effects caused by the inhibition of several neurotransmitters responsible for nociception transmission (33–35). The muscarinic receptors in the spinal cord participate in the mechanism of nociception suppression, as it has been proven that these receptors are responsible for the antinociception effects for anesthetic and analgesic drugs (36). Another pathway has been proposed to inhibit nociception by increasing the transport of noradrenaline. It has been found that Alfaxalone inhibits the mechanism of the reuptake of noradrenaline into the nerve cell by inhibiting the specific transporter of noradrenaline (37). Another mechanism was suggested to inhibit nociception for Alfaxalone and alfadolone in rats by activating the GABAA receptors in the spinal cord (33) and this supported by another study on rats using the tail-flick test, where it was found that alfadolone and not Alfaxalone have antinociceptive effects using sub-anesthetic doses of 10 mg/kg IP (38) that the unique anesthetic mechanism of Alfaxalone may be responsible for the analgesic effect. When matching the sedative properties of Alfaxalone with propofol in cats, there were no clear antinociceptive effects (38). The antinociceptive effect of the Alfaxalone observed by the previous research has shown that Alfaxalone has a distinct depressive effect on the sensation neuronal pathways of neurons in the dorsal cord of the cat's spinal cord (39). The specific antinociceptive impacts were eligible to ensure that the alfadolone portion of CT 1341 has been communicated and its act on the GABAA receptors in the central nervous system especially the spinal cord (33, 35, 39). Alfadolone has been found to cause antinociceptive effects without sedation in rodent, While sedation and anesthetics are caused by Alfaxalone without analgesic effect (38).

Conclusion

Alfaxalone at doses of 1, 2 and 4 mg/kg created dose-dependent antinociception in chicks by electro stimulator test. The 4 mg/kg dose rate produced an antinociceptive effect after 15 minutes of injection, these doses of Alfaxalone produced an antinociceptive effect by thermal method but not significant in comparison with the control group, like electrical method. The best time for analgesia was after 30 minutes in the two methods mentioned, surprise finding was the anti-inflammatory effect of Alfaxalone which needs more research to determine the exact mechanism for anti-inflammatory.

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Conflict of interest

The authors of this work certify that we have no conflict of interest.

Authors' contributions

ASN and AA have been assigned a prime role in planning, earning, statistical analysis, and interpreting results, wrote and have critically assessed the work for primary intellectual elements, confirmed the final view to be published.

Adherence to Ethical Standards

This study was reviewed by the head and members of the Scientific Committee of the Physiology, Biochemistry and Pharmacology Department at the College of Veterinary Medicine/University of Mosul, and the ethical approval was unanimously approved (approval number UM.VET.2021.2).

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