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Premedication and general anesthetic agents decrease plasma concentration of the endocannabinoid anandamide in dogs

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Objective
To determine the effect of premedication followed by isoflurane (ISO) versus sevoflurane (SEVO), length of general anesthesia (GA), and the amount of IV fluid administered on plasma endocannabinoid arachidonoyl ethanolamide (anandamide; AEA) concentrations in dogs undergoing GA.

Methods
This study was an analysis of samples collected during a previously designed prospective, randomized, single-blinded experimental study involving 21 client-owned dogs undergoing GA. Samples were collected from March through October 2021. Dogs were randomized to ISO or SEVO as the inhalant anesthetic. Blood samples collected before and after GA were used to measure plasma AEA concentrations using HPLC-MS-MS. Data included signalment, length of GA (minutes), surgery performed, fluid volume administered (milliliters per kilogram), and treatment with NSAIDs or steroids. Statistical analyses included power analysis, normality testing, and adjusted linear mixed models.

Results
Plasma AEA concentrations significantly decreased after GA in both groups. Least squares mean AEA concentration decreased from 29 to 12.3 ng/mL in the ISO group and from 26.6 to 11.1 ng/mL in the SEVO group. There were no significant differences between groups or associations with anesthesia duration, fluid volume, surgery, or NSAID/steroid use.

Conclusions
Plasma AEA concentrations were significantly reduced after GA in both the ISO and SEVO groups. This reduction may be influenced by other anesthesia agents, such as dexmedetomidine, hydromorphone, and propofol.

Clinical Relevance
This study is the first to highlight a potential interaction between premedication, GA, and endocannabinoid signaling. Further research is needed to explore these findings and their implications for pain management and neuroprotection.

Keywords: canine, endocannabinoid system, general anesthesia, isoflurane, sevoflurane

The endocannabinoid (EC) system (ECS) is a vital neuromodulatory network that plays a crucial role in regulating numerous physiological processes, including neuronal development, synaptic plasticity, immune function, and pain modulation.[1] Its interactions with both the CNS and the immune system are mediated primarily through 2 receptors, cannabinoid receptor 1 and cannabinoid receptor 2, which are activated by endogenous cannabinoids or ECs, such as arachidonoyl ethanolamide (anandamide; AEA) and 2-arachidonoyl glycerol (2-AG).[2–5]

Cannabinoid receptor 1 is abundantly found in the CNS, particularly in the cortex, basal ganglia, hippocampus, and cerebellum, specifically at the presynaptic nerve terminal, whereas cannabinoid receptor 2 is mainly found within the immune system but expressed at a much lower concentration in the CNS, particularly under certain pathological conditions.[6–9] The ECS plays a key role in controlling pain perception, neurotransmitter release, and inflammatory responses.[10,11] A wide range of animal studies[12–17] have confirmed the analgesic effects of cannabinoids in multiple pain models, including both acute and

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physiological pain, effectively reducing pain caused by thermal, mechanical, and chemical stimuli.

Despite the growing evidence of research on the ECS, its role in dogs undergoing general anesthesia (GA) with commonly used inhalant anesthetics, like isoflurane (ISO) and sevoflurane (SEVO), has not been investigated. Studies[18,19] have shown the interaction of GA with the ECS. For example, Patel et al[18] were the first to demonstrate such interaction, showing that propofol increases whole-brain AEA concentration in mice. This effect is believed to contribute to its sedative properties as propofol acts as a competitive inhibitor of fatty acid amide hydrolase (FAAH), the enzyme responsible for breaking down AEA.[18] Schelling et al[19] further demonstrated that GA with SEVO leads to a decline in AEA concentrations, whereas the use of propofol increased the AEA concentrations in humans.

Moreover, differences in anti-inflammatory and antioxidant properties have been observed between ISO and SEVO, with SEVO showing a greater ability to reduce the production of proinflammatory cytokines and promote anti-inflammatory responses.[20,21] These findings raise intriguing questions about the differential effects of these inhalant anesthetic agents on the ECS and may play a role in pain modulation and inflammation in dogs.

The growing evidence supporting the analgesic properties of cannabinoids, combined with the anti-inflammatory and antioxidant effects of SEVO compared to ISO, underscores the need to explore the interaction between anesthesia and the ECS further. The purpose of the study reported here aimed to (1) determine the effect of premedication followed by either ISO or SEVO on plasma AEA concentration in dogs undergoing GA and (2) investigate the effect of the duration of GA and the IV fluid amount on plasma AEA concentration in dogs. We hypothesized that (1) plasma AEA concentration would be lower in dogs anesthetized with SEVO compared to ISO and that (2) longer anesthesia duration and a greater volume of IV fluid administration would be associated with lower plasma AEA concentrations.

Methods

This study analyzed previously collected samples from a prospective, randomized, single-blinded study involving dogs undergoing GA at the Auburn University Bailey Small Animal Teaching Hospital.[21] The study was approved by the Auburn University IACUC (No. 2020-3833) and the Auburn University College of Veterinary Medicine Clinical Research Review Committee.[21] Samples were collected from March 2021 through October 2021. Samples collected were preserved at −80 °C after collection, allowing their later use in evaluating plasma AEA concentration.

Client-owned dogs presenting to the Auburn University Bailey Small Animal Teaching Hospital from March 2021 through October 2021 with suspected intervertebral disk disease (IVDD) were eligible for enrollment. Eligibility required that dogs undergo GA for MRI and potential surgery, with informed consent obtained from the owners before inclusion. Preanesthetic diagnostics, including a CBC, serum biochemistry profile, and urinalysis, were performed on all recruited dogs. Exclusion criteria included dogs weighing less than 2.0 kg, those with metabolic diseases or infection identified through physical examination or laboratory tests, and dogs with a reported history of hypertension or hypotension. Additionally, dogs with a history of diabetic mellitus, renal disease, endocrine diseases (eg, hyperthyroidism or hyperadrenocorticism), or recent infections were also excluded. There were no upper limits on weight or age for inclusion. However, the use of steroidal drugs or NSAIDs within 30 days was recorded.

An online randomization generator was used to assign recruited patients to receive either ISO or SEVO as their gas inhalant anesthetic using a blocked randomization method with a block size of 8. Patients were enrolled numerically and assigned an anesthetic gas upon enrollment. The investigators and clinicians involved in sample collection and medical decision-making were not blinded, but the laboratory technician measuring plasma AEA concentrations was blinded. A single investigator collected 5 mL of whole blood at the time of IV catheter placement prior to anesthesia and an additional 5 mL via routine phlebotomy from a peripheral vein immediately after the inhalant anesthesia vaporizers were turned off. From each collection, the whole blood was processed to harvest plasma using EDTA tubes. All plasma samples were stored at −80 °C until analysis in January 2024, with a storage time ranging from 27 to 34 months depending on the collection date.

The anesthetic protocol was standardized such that all dogs received an IM dose of dexmedetomidine at 0.006 mg/kg and hydromorphone at 0.1 mg/kg prior to induction. Propofol was administered IV and titrated to effect, with anesthesia maintained using either ISO or SEVO based on the randomized group. Lactated Ringer solution (LRS) was administered IV at approximately 5 mL/kg/h during anesthesia. Additional fluid boluses, analgesics, or synthetic catecholamines for blood pressure support were administered at the anesthesiologist's discretion based on individual patient needs.

Baseline data for each dog included signalment, body weight, anesthetic gas used, and MRI diagnosis. The use of corticosteroids or NSAIDs within the week prior to presentation was recorded. The monitoring of vital signs (blood pressure, temperature, heart rate, respiratory rate, and end-tidal CO_2) was conducted per hospital protocol by anesthesia personnel. The total anesthesia time was documented in minutes along with the total volume of LRS infused, measured in mL/kg.

Arachidonoyl ethanolamide measurement

Chemicals and reagents—Arachidonoyl ethanolamide and AEA-d_4 were purchased from Abcam. Arachidonoyl ethanolamide-d_8 was ordered from Cayman Chemical Company. High-performance liquid chromatography–grade acetonitrile and methanol were acquired from VWR Chemicals. High-performance liquid chromatography–grade formic acid was obtained

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from Fluka Analytical. Glacial acetic acid was purchased from EMD Millipore. Ultrapure water (> 18.3 mΩ) was obtained from a water purification system (PICOPURE 3; Thermo Fisher Scientific).

Liquid chromatography–tandem mass spectrometry analyses—Liquid chromatography was performed on an Agilent 1290 UPLC system with an autosampler and flow-controlled binary pump. The chromatographic separation was performed using a reverse-phase Waters ACQUITY UPLC Peptide CSH C18 Column (2.1 X 10 mm, 1.7 µm). The column temperature was controlled at 40 °C for improved chromatographic performance. The mobile phase consisted of 0.2% (weight per volume) acetic acid in water (solvent A) and 0.1% (volume per volume) formic acid in acetonitrile (solvent B). The gradient elution was optimized for separation using the following time: 75% solvent B was increased to 100% for the initial 3 minutes and returned to 75% solvent B for 4 minutes at a flow rate of 0.3 mL/min. The autosampler temperature was maintained at 4 °C, and the injection volume was 1.2 µL.

The detection was performed on an Agilent 6460 Triple Quadrupole mass spectrometer with an Agilent Jet Stream electrospray ionization source in positive-ion mode controlled by Agilent Mass Hunter Acquisition software (version B.06.00). Nitrogen was used as desolvation gas and collision gas. Desolvation and sheath gas temperatures were set at 300 °C and 400 °C, with a flow rate of 5 L/min and 10 L/min. The nebulizer was set at 50 psi, and the capillary voltage was 4,000 V with a chamber current of 0.2 µA. Multiple reaction monitoring scan mode was used for ion acquisition. The following transitions were monitored: m/z 348.22 to 61.99 (AEA), m/z 352.2 to 66.16 (AEA-d_4), and m/z 356.25 to 62.96 (AEA-d_8; the detailed multiple reaction monitoring transitions and conditions for each analyte are listed in **Table 1**).

Preparation of calibration standards and quality control samples—Arachidonoyl ethanolamide, AEA-d_8, and AEA-d_4 (internal standard) stock solutions were prepared in methanol at the concentrations of 10 µg/mL, 10 µg/mL, and 1 µg/mL, respectively. Stock solutions of analytes and their internal standards were stored in screw-cap amber vials at −20 °C until use. The stock solutions of AEA and AEA-d_8 were serially diluted with acetonitrile to generate working standard solutions from 19.53 to 5,000 ng/mL. Working solutions of deuterium-labeled surrogate analytes were used to create calibration curves, whereas endogenous analytes were used to determine the response factors necessary for accurate quantification. To prepare a calibration curve, 10 µL of the serially diluted working standards were spiked into blank canine plasma to obtain final concentrations of 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, and 200 ng/mL. Separately prepared quality control (QC) samples were prepared (n = 3) at low, middle, and high concentrations of 3.13, 25, and 100 ng/mL, respectively. All the calibration standards and QC samples were prepared freshly each day. The standard curve was linear with concentrations within 10% of their normal values, and QC samples were within 10% of their nominal values with coefficients of variation ≤ 15%.

Sample preparation—After collection, canine plasma samples were kept in polypropylene cryotubes at −80 °C until batch analysis. An aliquot of 100-µL plasma samples (calibration, QC, and test samples) was spiked with 10 µL of internal standard solution (1 µg/mL of AEA-d_4). Protein precipitation was performed by adding 890 µL of ice-cold acetonitrile into the samples and vortex mixing for 1 minute, followed by centrifuging at 14,000 X *g* for 15 minutes at 4 °C. After centrifugation, 400 µL of supernatant was collected into glass culture tubes and gently dried with nitrogen gas at 30 °C. The residue was resuspended in 100 µL of acetonitrile and transferred into HPLC vials with low-volume inserts for analysis.

Surrogate analyte assay—Due to the presence of endogenous AEA in dog plasma, a surrogate assay approach was used to perform quantification using a published assay with slight modification.[22] Briefly, AEA-d_8 served as the surrogate analyte for calibration curve preparation, and AEA-d_4 served as the internal standard. The response factor (RF) was established by analysis of 4 replicated samples of surrogate analyte (AEA-d_8) and endogenous analyte (AEA) in neat acetonitrile solution at each standard concentration level. To determine ionization efficiency differences between the surrogate and endogenous analytes, the response factor was

Table 1—Summary of mass transitions and optimized parameters used for quantification and confirmation of anandamide (AEA), AEA-d_8, and internal standard, AEA-d_4.

Compound name	Precursor ion $[M+H]^+$	Product ion $[M+H]^+$	Dwell time (ms)	Fragment voltage (V)	Collision energy (eV)	Cell accelerator voltage (V)	Polarity
AEA (quantifier)	348.22	61.99	200	100	19	8	Positive
AEA (qualifier)	348.22	44.28	200	100	1	8	Positive
AEA-d_4 (quantifier)	352.2	66.16	200	120	25	8	Positive
AEA-d_4 (qualifier)	352.2	48.2	200	120	65	8	Positive
AEA-d_8 (quantifier)	356.25	62.96	200	120	19	8	Positive
AEA-d_8 (qualifier)	356.25	44.15	200	120	52	8	Positive

The precursor and product ion transitions were optimized by direct injection and analysis by ultra-high pressure liquid chromatography electrospray-ionization tandem mass spectrometry. Mass transitions were determined, and tandem mass spectrometry parameters (dwell time, fragment voltage, collision energy, cell accelerator voltage, and polarity) were optimized in multiple reaction monitoring scan mode for ion acquisition and used for the quantification of plasma AEA concentrations for 21 client-owned dogs immediately before and after undergoing general anesthesia with isoflurane or sevoflurane in a study conducted from March 2021 through October 2021.

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calculated by dividing the response area of the surrogate analytes by that of the endogenous analytes with the following equation:

$$RF = \frac{\text{Mean area of surrogate analyte}}{\text{Mean area of endogenous analyte at equivalent concentration}}$$

Using a surrogate assay approach, method validation was performed by evaluating linearity, sensitivity, accuracy, and precision **(Supplementary Tables S1–S3)**.

Statistical analysis

The power analysis was performed using G*Power (version 3.1.9.4, open source, free software available at https://www.psychologie.hhu.de/arbeitsgruppen/allgemeinepsychologie-und-arbeitspsychologie/gpower), with the α level at 0.05, power at 80%, and SD of 30% with a mean reported on the published data in humans. This resulted in 10 per group needed.[19] Probability and residual plots were generated to verify that data followed a normal distribution. Furthermore, the normality of the data was evaluated using the Kolmogorov-Smirnov test. Arachidonoyl ethanolamide concentrations were analyzed using repeated measures ANCOVA. The adjusted linear mixed model included treatment and time and the interaction between treatment and time as a fixed effect. Gender and age were included as random variables. Variables determined not to have been significantly associated with AEA concentrations were removed from the model using a backward selection process. Data were analyzed using R for Mac (version 4.2.2; R Core Team) and Prism, version 10.0 (GraphPad Software Inc), with an overall α set at $P < .05$.

Results

A total of 31 dogs' samples were initially available; however, 10 were excluded due to the following reasons: incorrect inhalant use (1), cardiopulmonary arrest (2), incomplete data (6), and absence of AEA-d_8 in the SEVO group (1). Therefore, data were available for 21 patients, with 13 dogs in the ISO group and 8 dogs in the SEVO group.

Demographics

The median (minimum to maximum) age was 5.5 (1 to 13) years. Of the included dogs, 2 were intact females (9.5%), 7 were spayed females (33.3%), 2 were intact males (9.5%), and 10 were neutered males (47.7%).

Isoflurane group

The median (minimum to maximum) age was 6 (2 to 13) years. Of the included dogs, 1 was an intact female (7.7%), 3 were spayed females (23.1%), 2 were intact males (15.4%), and 7 were neutered males (53.8%). The breeds represented were Dachshund (3 of 13 [23.1%]), mixed breed (3 of 13 [23.1%], Labrador Retriever (2 of 13 [15.4%]), Shih Tzu (2 of 13 [15.4%]), and 1 each of the following breeds: Vizsla, French Bulldog, and Basset Hound.

Sevoflurane group

The median (minimum to maximum) age was 5 (1 to 11) years. Of the included dogs, 1 was an intact female (12.5%), 4 were spayed females (50%), and 3 were neutered males (37.5%). The breeds represented were American Bulldog, Yorkshire Terrier, Bloodhound, Labrador Retriever, Dachshund, French Bulldog, Cocker Spaniel, and mixed breed; each accounted for 1 (12.5%).

Diagnosis and surgery

Based on the MRI findings, the diagnoses for the 21 dogs were IVDD (15 of 21 [71.4%]), fibrocartilaginous embolism or acute noncompressive nucleus pulposus extrusion (2 of 21 [9.5%]), neoplasia (2 of 21 [9.5%]), traumatic hemorrhage (1 of 21 [4.8%]), and nonspecific myelopathy (1 of 21 [4.8%]). Twelve of 15 dogs with IVDD underwent surgery, and of those, 8 were in the ISO group, and 4 were in the SEVO group. There was no significant difference in the diagnoses or surgical rates between the ISO and SEVO groups.

Volume of fluids administered and duration of anesthesia

All dogs received LRS IV fluid therapy throughout the GA. The median (minimum to maximum) amount of balanced crystalloid fluid received in the ISO group was 19.82 mL/kg (6.98 to 33.58) and in the SEVO group was 22.63 mL/kg (8.2 to 67.67). There was no significant difference in the volume of fluid administered between the ISO and SEVO groups. The median (minimum to maximum) anesthesia time in the ISO group was 327 minutes (130 to 452 minutes) and in the SEVO group was 245 minutes (139 to 493 minutes). There was no significant difference in the duration of anesthesia (minutes) between the ISO and SEVO groups.

Treatment received

Of the 21 dogs included in the study, 12 dogs (57%) received either an NSAID or a steroid in the week leading up to presentation, whereas 9 (43%) did not. Within the SEVO group, 6 of 8 (75%) had received an NSAID or steroid compared to 6 of 13 (46.15%) in the ISO group. There was no significant difference in the treatment received between the ISO and SEVO groups.

Plasma AEA concentration

The volume of fluids administered (milliliters per kilogram), duration of anesthesia (minutes), surgery (yes or no), and anti-inflammatory therapy (yes or no) were not significantly associated with AEA concentrations. Least squares mean AEA concentration significantly decreased after anesthesia for both ISO and SEVO treatment. Specifically, the least squares mean AEA concentration for the ISO group was 29 ng/mL before GA, with an SE of 3.03, which decreased significantly to 12.3 ng/mL after GA,

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with an SE of 3.02 (*P* < .001). Similarly, for the SEVO group, the least squares mean AEA concentration decreased from 26.6 ng/mL before GA (SE, 3.76) to 11.1 ng/mL after GA, with an SE of 3.74 (*P* < .001). However, there was no significant difference in AEA concentration between the 2 types of anesthesia both before and after their administration (*P* > .05).

Discussion

In this study, we investigated the effects of 2 commonly used inhalant anesthetics, ISO and SEVO, on plasma AEA concentrations in dogs undergoing GA for MRI. To the authors' knowledge, this is the first study to evaluate whether these anesthetics modulate plasma AEA concentration in dogs. We hypothesized that (1) plasma AEA concentrations would be lower in dogs anesthetized with SEVO compared to ISO following premedication and that (2) longer anesthesia duration and a greater volume of IV fluid administration would be associated with lower plasma AEA concentrations.

Our findings revealed that plasma AEA concentrations were significantly reduced after GA in both the ISO and SEVO groups, with no notable differences observed between the 2 anesthetic agents. These results contradict our first hypothesis, which anticipated a greater reduction in plasma AEA concentrations with SEVO compared to ISO. Similarly, no significant associations were observed between plasma AEA concentrations and anesthesia duration, IV fluid volume, surgery, or NSAID/steroid use, contrary to our second hypothesis.

Interestingly, the findings suggest a complex interplay between the anesthetic protocol and plasma AEA concentrations, with potential contributions from premedications (hydromorphone and dexmedetomidine), the induction agent (propofol), or the inhalant anesthetics (ISO and SEVO). While previous studies[18,19] have demonstrated specific effects of anesthetic agents on the ECS, direct comparisons with our findings are limited due to differences in study design and anesthetic agents evaluated.

The process through which GA triggers a reduction in plasma EC concentration is not yet understood. Research indicates that EC signaling increases in both the brain and peripheral tissues in response to stress and physical exercise.[23–25] This suggests a possible hypothesis that the observed decline in plasma AEA concentration following anesthesia induction may result from a reduction in nonspecific stress associated with the anesthetic protocol. Since AEA synthesis is known to be regulated by various factors, including catecholamine release and α-adrenergic receptor activation, it is possible that anesthesia may indirectly modulate ECS activity through these pathways.[26,27]

The influence of our anesthetic protocol must be considered as these agents are known to interact with stress and pain pathways, which are closely linked to the ECS. For instance, α-2 adrenergic receptor agonists, such as xylazine, have been shown to induce peripheral antinociception through the release of AEA and subsequent activation of cannabinoid 1 receptors.[28,29] Similarly, research on opioids, particularly morphine, has shown that their analgesic effects can involve ECS activation, particularly through the cannabinoid 1 receptor–mediated pathway.[30] Finally, propofol, a commonly used induction agent, acts as a competitive inhibitor of FAAH, the enzyme responsible for AEA degradation.[18] These considerations highlight the importance of future studies designed to isolate the effects of individual anesthetic components of ECS activity.

Interestingly, while SEVO has been shown to possess stronger anti-inflammatory and antioxidant properties compared to ISO, this did not translate into a significant difference in plasma AEA concentrations between these 2 inhalant anesthetic agents in our study.[20,21] This suggests that the impact of these anesthetics may be independent of their known anti-inflammatory effects. Additionally, the lack of association between plasma AEA concentration and volume of fluid administered, duration of anesthesia, surgery, and steroid/anti-inflammatory therapy used implies that other factors may be at play in modulating ECS during anesthesia.

This study had several limitations. The findings of the study cannot conclusively attribute changes in AEA solely to inhalant anesthetics, and the role of other anesthetic agents may have also contributed to the change. Second, the power analysis conducted prior to the study indicated that a sample size of 10 dogs/group would be required. However, due to dropout due to incorrect inhalant use, cardiopulmonary arrest, and incomplete data, the final sample size consisted of 13 dogs in the ISO group and 8 dogs in the SEVO group. While the study still provides valuable insights, the reduced sample size may affect the overall power and ability to detect more subtle differences between the groups. Third, the reduction in plasma AEA concentration was attributed to the anesthetic agents, but stress levels were not directly measured, which would have strengthened the hypothesis that the decrease in plasma AEA is related to stress reduction. Additionally, some patients received intraoperative hydromorphone as needed for analgesia, which was not standardized across all cases. This variability in opioid use may have influenced plasma AEA concentrations and represents an additional confounding factor. Furthermore, the plasma AEA concentration was only measured at a single time point, providing a limited view of its fluctuations over time. The study did not evaluate other important endogenous system components, such as 2-AG and FAAH concentration, which could provide a more comprehensive understanding of the effects of anesthesia on the ECS. The accurate measurement and interpretation of 2-AG concentrations can be challenging as accurate measurement requires baseline chromatographic separation from 1-AG, an isomer with identical collision-induced disintegration spectra and mass transition.[31] Besides that, the dose of propofol required for anesthesia induction can vary between individuals, which may have influenced the observed outcomes, and the mean end-tidal concentrations of ISO and SEVO could further

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provide insight into potential anesthetic depth variations among the groups. Finally, the potential direct interactions between sex hormones and the ECS were not accounted for in this study due to the limited sample size.[32]

Given the role of the ECS in modulating pain perception, neurotransmitter release, and anti-inflammatory responses, the observed reduction in plasma AEA concentration may have implications for pain management and neuroprotection in dogs undergoing anesthesia. Further research is needed to clarify the mechanisms by which individual anesthetic agents interact with the ECS and to explore potential therapeutic strategies targeting the ECS for improved anesthetic outcomes.

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Disclosures

The authors have nothing to disclose.

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Supplementary Materials

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