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Safety Evaluation of a Ketamine–Dodecyl Maltoside Combination Using Angiogenesis and Embryonic Development Models

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Abstract

Ketamine (KET) exhibits potential anticancer activity, with enhanced cytotoxicity against melanoma cells when combined with the permeation enhancer dodecyl maltoside (DDM). To support clinical translation, this study evaluated the embryotoxicity, effects on angiogenesis, and cytocompatibility of KET, DDM, and their combination (KET+DDM) using a chicken embryo model. The chorioallantoic membrane (CAM) assay was used to assess angiogenesis following treatment with KET (1000 μM), DDM (19.6 μM), or KET+DDM in fertilized eggs. Embryonic survival and morphology were monitored for five days. Quantitative PCR analysis of heart, lung, kidney, and brain tissues evaluated apoptosis-related genes (Caspase 3, Caspase 8, Caspase 9, BAX) and VEGF expression. Cytocompatibility was examined in primary embryonic fibroblasts (EFBs) using AlamarBlue assays and morphological assessment. The results showed no significant differences in vascular density, vessel length, or branching in the CAM assay across all treatments. Embryos treated with KET or KET+DDM showed normal survival and morphology, while DDM alone reduced viability. Apoptotic and angiogenic gene expression remained unchanged in major organs. *In vitro*, KET and KET+DDM did not reduce EFB viability or alter morphology. Overall, KET and KET+DDM did not produce detectable adverse effects in embryonic and cellular models, preserving angiogenesis and development. Notably, the KET+DDM combination showed no detectable adverse effects, supporting the preliminary safety of this combination for further anticancer investigation.

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Keywords: Ketamine, dodecyl maltoside, angiogenesis, embryogenesis, chorioallantoic membrane (CAM), cell viability.

1. Introduction

Ketamine (KET), a widely used anesthetic and N-methyl-D-aspartate (NMDA) receptor antagonist, has recently attracted interest for its emerging anticancer properties beyond its established clinical applications (1). Several studies have shown that KET could modulate intracellular calcium signaling, interfere with proliferative pathways, and induce apoptosis in a dose-dependent manner in multiple cancer types, including lung, gastric, pancreatic, and liver cancers (1–7) In our recent work, KET exhibited enhanced cytotoxicity against melanoma cells when combined with dodecyl maltoside (DDM), resulting in a significant reduction in cell viability (8). This combination is being explored as a formulation-based strategy aimed at enhancing intracellular delivery and therapeutic selectivity of KET toward cancer cells. DDM is a non-ionic surfactant extensively employed as a permeation enhancer to improve drug transport across biological barriers, including the skin, owing to its ability to transiently modulate membrane permeability (9–13). Despite its safety profile and widespread use, surfactant-based permeation enhancers might interact with normal cellular membranes, raising concerns regarding their potential effects on non-target tissues and developmental processes (14–18).

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The evaluation of drug safety and biocompatibility is a critical step in the development of novel or repurposed therapeutic strategies, particularly for formulations that incorporate permeation enhancers (19,20). Unintended interactions with normal biological processes, including vascular development and tissue integrity, might result in adverse effects that limit clinical translation. Physiological angiogenesis represents a sensitive indicator of tissue health and normal development, as it reflects the integrity of endothelial function and vascular organization (21,22). Because angiogenesis and apoptotic signaling are fundamental both to tumor biology and to normal embryonic development, developmental angiogenic models provide a sensitive platform for detecting off-target or formulation-related toxicity. However, alterations in this process, whether excessive or inhibitory, might signal underlying toxicity rather than therapeutic benefit. In cancer, cells promote angiogenesis to support their survival, growth, and metastasis (23). This process is driven by growth factors from cancer and stromal cells, leading to abnormal, leaky vasculature that facilitates tumor progression. (24,25). In addition to vascular assessment, embryonic viability and cellular compatibility are critical parameters for establishing the overall safety profile of drug combinations (26,27). Consequently, understanding how KET+DDM formulation interacts with the early stage of the embryo, including its blood vessel development, provides valuable insight into its safety and biological tolerability.

The chicken embryo chorioallantoic membrane (CAM) model is a robust, ethically accessible, and highly visual system for evaluating developmental safety, vascular integrity, and cytocompatibility effects (28). Its rapidly expanding, naturally vascularized membrane during mid-embryogenesis makes it ideal for visualizing angiogenesis and detecting vascular alterations (26,29). The model allows real-time observation, controlled compound application, and quantitative assessment of vascular parameters such as vessel density and branching, while also supporting studies of embryotoxicity

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and early developmental responses to chemical exposure (30,31). Although the proposed KET+DDM combination has shown measurable anticancer activity against melanoma cells (8), its impact on angiogenesis and early developmental processes remains insufficiently explored. Accordingly, the present study employed the CAM model to evaluate the safety of KET, DDM, and their combination by assessing their effects on physiological vascular development, determine embryonic tolerability during early stages of development, and assess cytocompatibility through analysis of cell viability in primary embryonic fibroblasts.

2. Materials and methods

2.1 Materials

Ketamine (KET) was obtained from Supriya Lifescience Ltd., Mumbai, India, while dodecyl maltoside (DDM; CAS 6922-93-6) was sourced from Avanti Polar Lipids, USA. All additional reagents were of analytical grade and used according to the manufacturer's instructions.

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2.2 Methods

2.2.1 Chicken embryos

Fertilized White Leghorn eggs (Mazzraty Poultry, Qatar) were incubated in a MultiQuip incubator at 37 °C and 60% relative humidity. To minimize adhesion of the embryo to its surrounding membranes, eggs were rotated every hour (32). For the angiogenesis study, five-day-old embryos were used for the treatment, while three-day-old embryos were used for embryogenesis assessment. Treatments were administered according to our previously established protocols (8,33). Treatment solutions were freshly prepared in a total volume of 1 mL by adding 10 µL of the respective stock solution to 990 µL of sterile PBS to achieve the desired final concentrations (KET: 1000 µM; DDM: 19.60 µM; KET+DDM: 1000+19.60 µM), followed by gentle mixing. In brief, the 10 µL was carefully applied dropwise onto the coverslip, then positioned over the CAM surface, ensuring localized and uniform exposure

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while minimizing mechanical disturbance to the membrane. Control embryos received an equivalent volume of PBS. The selected concentration was based on prior in vitro studies, where it produced measurable biological effects near the IC_{50}, and was applied as a single-dose screening to sensitively detect potential embryonic or angiogenic perturbations and interaction effects. For the angiogenesis assessment, images were captured 48 h after treatment and analyzed using AngioTool Software (version 0.6a) following the method described by Zudaire et al. (34). AngioTool analysis was performed using automated quantitative software to minimize operator-dependent variability. Although formal blinding was not implemented, all images were analyzed using identical software parameters and predefined regions of interest (ROI) to ensure objective and standardized quantification. For the embryogenesis analysis, embryos were dissected five days after dosing, and tissues including the heart, lungs, kidneys, and brain were collected for RNA extraction and subsequent qPCR analysis.

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2.2.2 RNA Extraction and qPCR

Total RNA was isolated from heart, lungs, kidneys, and brain tissues that were collected from chicken embryos using the NucleoSpin TriPrep Mini kit (MACHEREY-NAGEL, Germany), following the manufacturer's protocol. RNA concentration was determined using a Nanodrop spectrophotometer (Thermo-Fisher Scientific, USA), and purity was assessed using the 260/280 nm absorbance ratio, with values near 2.0 indicating high-quality RNA. For each tissue, RNA was extracted from separate embryos, and three independent biological replicates per treatment group (n = 3) were analyzed; no pooling of samples was performed. Complementary DNA (cDNA) was synthesized using the SuperScript™ III First-Strand Synthesis SuperMix kit (Thermo Fisher Scientific, USA) following the manufacturer's protocol. Quantitative PCR was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Australia) on a QuantStudio® 5 Real-Time PCR System to assess expression of apoptosis- and angiogenesis-related genes, including

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Caspase 3, Caspase 8, Caspase 9, VEGF, and BAX. Gene expression was normalized to the housekeeping gene GAPDH, and relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Primer specificity was confirmed by melt-curve analysis, which demonstrated a single distinct peak for each target gene. All primer sequences used in this study are provided in table 1.

Table 1. Information of specific primers used for the amplification in PCR.

Primer	Sequence
Caspase 3	F: 5'- GATCAGGACGAGCAGGACG -3'
	R: 5'- TGTCCAGATGCCACAGTTC -3'
Caspase 8	F: 5'- TGAGTACGCTGTTTGCTCTG -3'
	R: 5'- CTTGACGTTGGGTTGACTTG -3'
Caspase 9	F: 5'- CAGCGTGTTTGTCCTGACTG -3'
	R: 5'- TCTGAGGCGTGACCTGAGTG -3'
BAX	F: 5'-TCACAGCCAGGAGAATCGCAC -3'
	R: 5'-GCTGCAGACATGCTGTGGATC -3'
VEGF	F: 5'- AGCCTCCTCCTGGTGCTTCT -3'
	R: 5'- TGTGATGATTGCTGCTTGTG -3'
GAPDH	F: 5'- GAAGGTGAAGGTCGGAGTC -3'
	R: 5'- GAAGATGGTGATGGGATTTC -3'

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2.2.3 Cell culture

The effect of KET, DDM, and their combination (KET+DDM) treatments was evaluated using primary embryonic fibroblasts (EFBs) obtained from the embryos as previously described (35). The EFBs were generated in our laboratory from 10-day-old chicken embryos. In summary, embryos were

carefully removed from the eggs, after which their limbs and internal organs were dissected. The remaining tissue was then processed through several rounds of digestion using 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA) and phenol red (Gibco, Life Technologies). EFBs were cultured in complete growth medium, Gibco® RPMI-1640 (Roswell Park Memorial Institute-1640) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Life Technologies) and 1% PenStrep (Invitrogen, Life Technologies). Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. All experiments were carried out once the cultures reached approximately 70%–80% of confluency.

2.2.4 Cell Viability Assay

Primary EFBs were plated in 96-well plates (Thermo Fisher Scientific, USA) at a density of 10,000 cells per well and allowed to adhere overnight. Following this, the cells were exposed to different concentrations of KET (0, 1, 10, 100, 500, 1000, 1500, and 2000 µM), DDM (1.96, 4.90, 9.79, and 19.60 µM), and a combination treatment was applied, where cells were exposed to different concentrations of KET (0, 1, 10, 100, 500, 1000, 1500, and 2000 µM) in the presence of 19.60 µM DDM for a period of 48 h, with untreated cells serving as controls. Cell viability was evaluated using the AlamarBlue™ Cell Viability Reagent (Invitrogen, Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Fluorescence measurements were obtained at an excitation wavelength of 560 nm and an emission wavelength of 600 nm using an Infinite m200 PRO fluorescent microplate reader (TECAN, Switzerland). Potential interference of KET and DDM with the AlamarBlue assay was evaluated by testing each compound at the assay's excitation/emission wavelengths (560/600 nm); neither compound exhibited absorbance or fluorescence under these conditions, confirming that the measured signal reflects cellular metabolic activity without compound-related interference.

2.2.5 Ethical Statement

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All experiments involving chicken embryos were conducted following approval and guidelines from the Institutional Bio-Safety Committee of Qatar University (QU-IACUC 013/2024). All methods were performed in accordance with the relevant guidelines and regulations by the committee.

2.2.6 Statistical Analysis

Data are expressed as the mean ± standard deviation (SD) from independent experiments (n = 3). Statistical comparisons between treated and untreated cells were performed using one-way ANOVA, two-way ANOVA, Šidák's multiple comparisons test, and Dunnett's test, all conducted in GraphPad Prism 10 software. The two-way ANOVA was performed using treatment group (Control, KET, DDM, and KET+DDM) and gene type (Caspase 3, Caspase 8, Caspase 9, BAX, and VEGF) as the two factors. Levels of statistical significance are indicated as follows: ns (not significant), $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

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3. Results

3.1 Effects of KET and DDM on Angiogenesis

In this work, 5-day-old chicken embryos were exposed to KET, DDM, and their combination (KET+DDM) for a period of 48 h. Following previously established protocols (33,35,36), a round coverslip was placed directly over the CAM to enable direct comparison between treated and untreated regions. Examination of the CAM showed that the formation of fine, interconnected capillaries remained largely consistent across all treatment groups in comparison to the control, with no visible disruption or reduction in vascular density (Figure 1A). The dense vascular network observed in control embryos was preserved in embryos treated with KET, DDM, or the KET+DDM combination. Further, quantitative analysis using AngioTool software supported these observations, revealing no significant differences ($p > 0.05$) in vessel area, vessel percentage area, total number of junctions, junctions

density, total vessel length, and total number of end points among any of the groups compared to the control (Figure 1B). These results indicate that, under the conditions tested, KET and DDM either alone or in combination did not induce detectable changes in the analyzed morphological angiogenic parameters in the CAM model. Overall, the findings demonstrate that the KET+DDM formulation did not produce detectable adverse effects in a physiological embryonic context and does not elicit antiangiogenic effects, underscoring its potential safety for further preclinical studies.

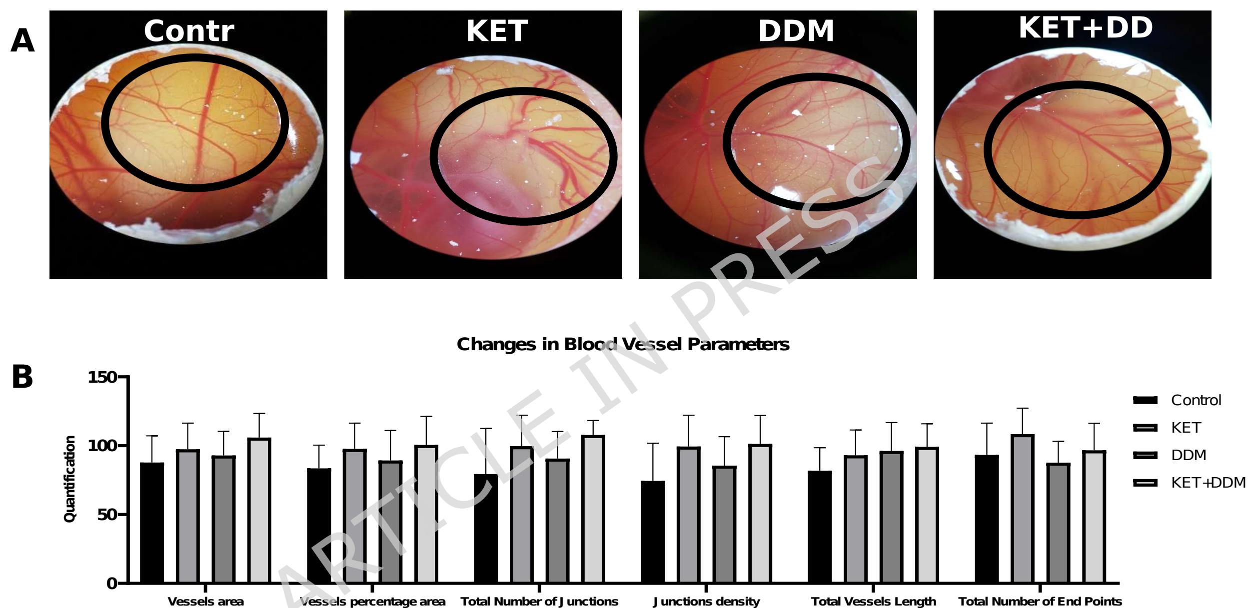

Figure 1. CAM angiogenesis assay in chicken embryos. Chicken embryos were treated with KET, DDM, and their combination (KET+DDM). **(A)** Representative stereomicroscopic images illustrating the effects of KET, DDM, and KET+DDM on angiogenesis in the CAM. Images were acquired 48 h post-treatment using a stereomicroscope (n = 3). **(B)** Quantitative analysis of angiogenesis showing vessel area, vessel percentage area, total number of junctions, junctions density, total vessel length, and total number of end points following treatment with KET, DDM, and KET+DDM. The data are presented as mean ± SD (n=3, total number of embryos per group=20). Statistical analysis was performed using a 1-way ANOVA, and Tukey's post-hoc test **Abbreviations**: KET: ketamine; DDM: dodecyl maltoside; CAM: Chorioallantoic membrane; SD: Standard deviation; ANOVA: Analysis of variance.

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3.2 Effects of KET and DDM on Normal Embryonic Development

A 3-day-old chicken embryo model was employed to evaluate the embryotoxicity of KET, DDM, and their combination (KET+DDM) following five days of exposure. The results demonstrated no significant embryotoxicity upon treatment with KET or the KET+DDM combination compared to the untreated control ($p > 0.05$) (Figure 2A). Consistently, no significant reduction in embryonic survival was observed by the fifth day of exposure, indicating that both KET alone and the KET+DDM formulation did not show detectable adverse effects during the early stages of normal embryonic development ($p > 0.05$). In contrast, treatment with DDM alone resulted in a significant decrease in embryo viability starting from day 2 compared to the control group (Figure 2A).

To further investigate potential molecular effects, major organs including the heart, lungs, kidneys and brain were harvested from treated and untreated embryos for quantitative PCR analysis of key genes associated with apoptosis and morphological parameters of angiogenesis, namely Caspase 3, Caspase 8, Caspase 9, BAX, and VEGF (Figure 2B–2E). The analysis revealed that apoptosis-related gene expression did not show widespread significant alterations in embryos treated with KET, DDM, or their combination compared to controls. However, specific differences were observed, including a significant change in Caspase 3 expression in the DDM-treated group compared to the control group (adjusted $p = 0.0204$), and a significant change in BAX expression in the KET-treated group compared to the control group (adjusted $p = 0.0486$). No significant differences were detected for Caspase 8 or Caspase 9 across treatment groups ($p > 0.05$). Moreover, no significant changes in VEGF expression were detected across all examined tissues, suggesting that angiogenic signaling remained unaffected under the tested conditions. In agreement with these molecular findings, gross morphological assessment showed no observable

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abnormalities in embryo growth or development following treatment with KET, DDM, or the KET+DDM combination (Figure 3). Overall, these results indicate that while limited gene-specific changes in apoptosis markers were observed, there was no evidence of a consistent or robust activation of apoptotic or angiogenic pathways, and no adverse effects were detected for KET and its combination with DDM at the embryonic and molecular levels under the tested conditions.

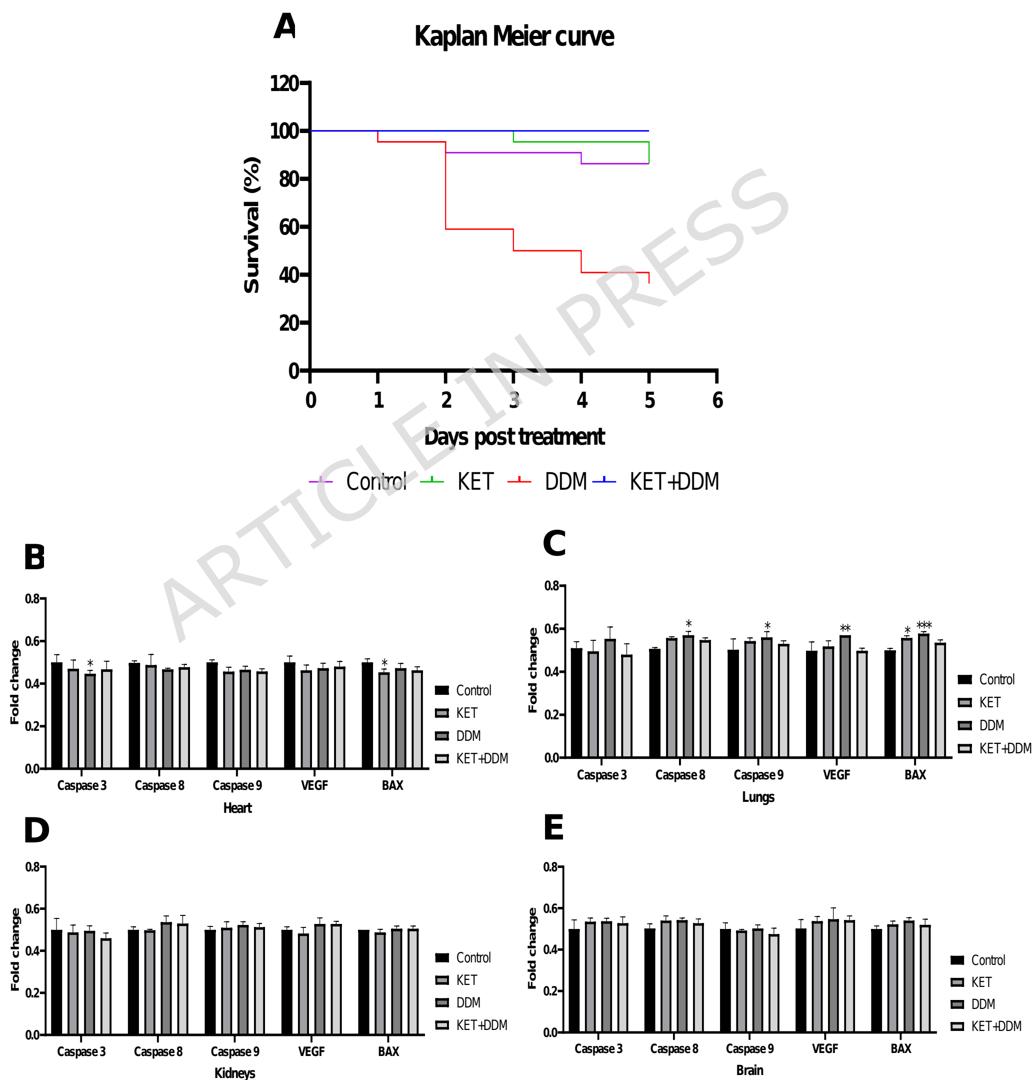

Figure 2. Effects of KET, DDM, and their combination (KET+DDM) on embryogenesis. **(A)** Kaplan–Meier survival curve comparing the survival rates of chicken embryos treated with KET (1000 µM), DDM (19.60 µM), and their combination (KET+DDM, 1000+19.6µM), over a five-day period post-treatment. (B–E) qPCR analysis displaying the changes in gene expressions of the tested apoptotic and angiogenic markers in tissues derived from the treated/untreated chicken embryos, namely **(B)** heart, **(C)** lungs, **(D)** kidneys, and **(E)** brain tissues. KET and its combination (KET+DDM) treatment induced no significant downregulation of genes associated with apoptosis and VEGF compared to the controls. The data are presented as mean ± SD (n=3, total number of embryos per group=20). Statistical analysis was performed using 2-way ANOVA and Šidák's test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate statistical significance compared to the control). The two-way ANOVA applied to panels (B–E) was performed using treatment group (Control, KET, DDM, and KET+DDM) and gene type (Caspase 3, Caspase 8, Caspase 9, BAX, and VEGF) as the two factors. **Abbreviations**: KET: ketamine; DDM: dodecyl maltoside; qPCR: Quantitative real-time polymerase chain reaction; VEGF: Vascular endothelial growth factor; SD: Standard deviation; ANOVA: Analysis of variance.

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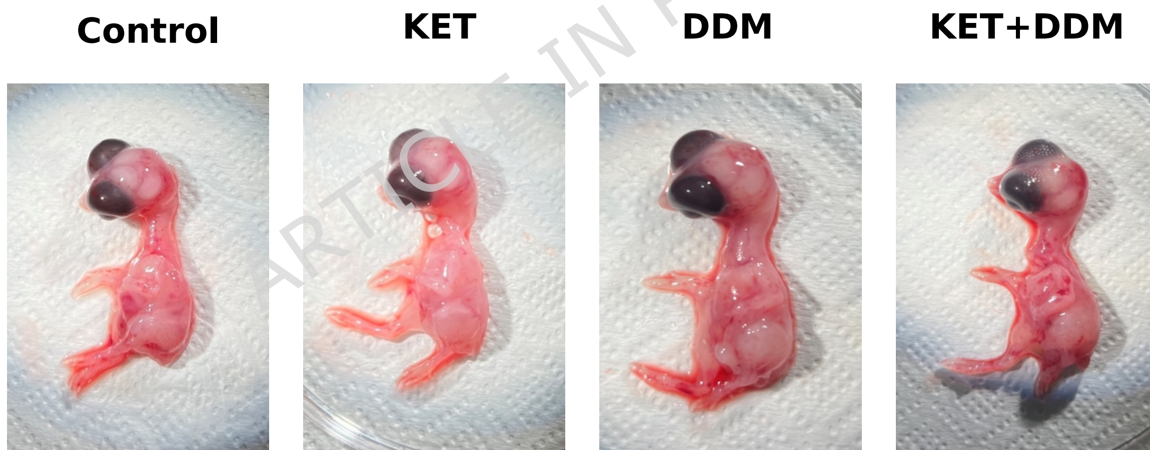

Figure 3. Post-morphological assessment of chicken embryos treated with KET (1000 µM), DDM (19.60 µM), and their combination (KET+DDM, 1000+19.6 µM). Images were taken post day 5 of treatment (n=3, total number of embryos per group=20).

3.3 Effects of KET and DDM on cell viability

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To assess their effects on cell viability, primary EFBs were treated with KET, DDM, and their combination (KET+DDM) for 48 h. Notably, no statistically significant reduction in cell viability was observed across all treatment groups compared to the untreated control ($p > 0.05$) (Figure 4A), suggesting no detectable adverse effects on normal cells under the tested conditions. Consistent with these findings, morphological examination further confirmed the lack of any noticeable decrease in cell number or viability, as well as the lack of observable alterations in cellular morphology following treatment (Figure 4B). The treated EFBs maintained their typical fibroblast-like morphology and normal growth patterns, comparable to control cells. Overall, these results demonstrate that both KET alone and the KET+DDM combination did not exert any measurable adverse effects on primary EFBs under the tested conditions, supporting their biocompatibility and preservation of normal cellular growth and proliferation.

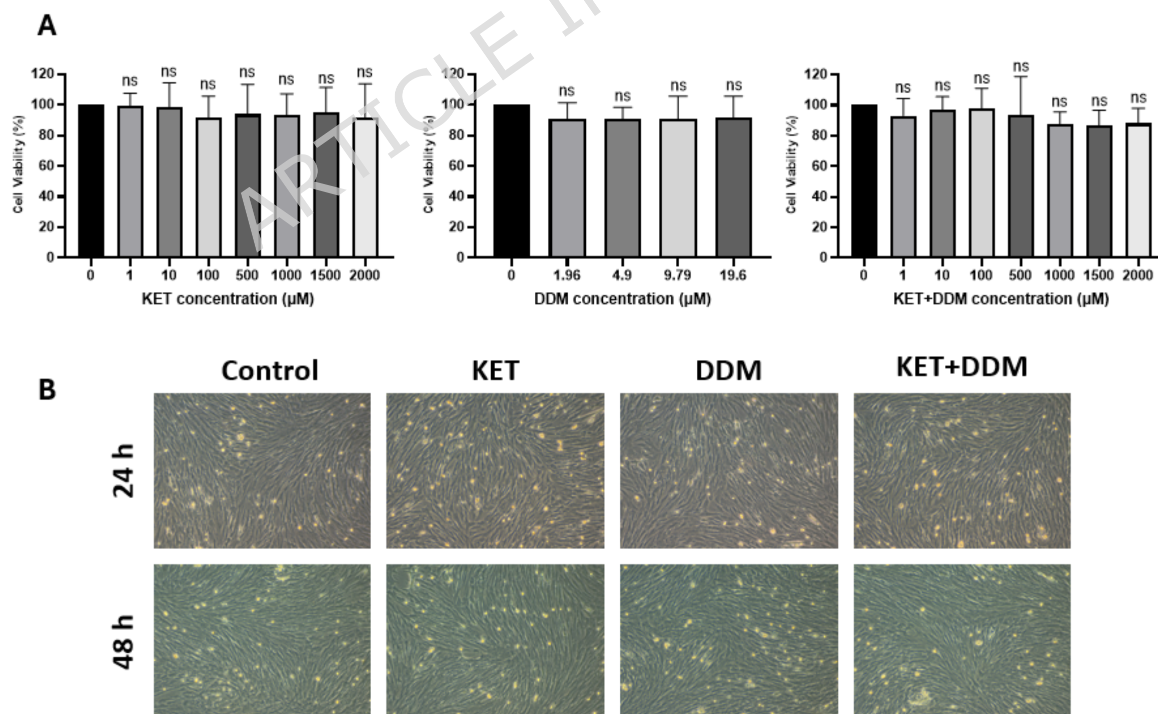

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Figure 4. The effect of KET, DDM, and their combination (KET+DDM) on **(A)** the cell viability and **(B)** morphology of primary EFBs. **(A)** Effect of KET, DDM, and their combination (KET+DDM) on cell viability of EFBs after 48 h. No significant reduction in cell viability was observed in all treatment groups. **(B)** The images show no significant morphological changes in cells across the KET (1000µM), DDM (19.60 µM), and their combination (KET+DDM, 1000+19.6µM) treatments, indicating its safety on EFBs. Images were taken at a magnification of 10× following 48 h of treatment (n = 3). The data are presented as mean ± SD (n=3). Statistical analysis was performed using 1-way ANOVA and Šidák's test (ns: not significant). **Abbreviations**: KET: ketamine; DDM: dodecyl maltoside; EFBs: Embryonic fibroblasts, SD: Standard deviation.

4. Discussion

This work provides a comprehensive *in ovo* and *in vitro* safety evaluation of KET, DDM, and their combination using the CAM model and primary embryonic fibroblasts. This work was motivated by our previous findings demonstrating an enhanced anticancer activity of the KET+DDM combination against melanoma cells, where DDM was proposed to improve cellular permeation and potentiate KET's cytotoxic effects (8). Given the central role of angiogenesis and embryonic development in toxicity assessment, the current study aimed to determine whether this combination elicits unintended antiangiogenic, embryotoxic, or cytotoxic effects in normal physiological systems.

One of the key findings was that KET, DDM, and their combination did not disrupt morphological parameters of angiogenesis in the CAM model. The CAM assay is a well-established, highly sensitive platform for detecting both pro- and antiangiogenic effects, and is frequently used for early safety screening of anticancer agents (27,37). Quantitative AngioTool analysis demonstrated vascular parameters, including vessel area, vessel percentage area, total number of junctions and its density, total vessel length, and total number of endpoints remained unchanged across all treatment groups

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compared to controls. These findings suggest that, at the tested concentrations, KET does not exert antiangiogenic effects under physiological conditions, either alone or when combined with DDM. This finding is important because while KET is being considered for anticancer use, inhibition of normal angiogenesis could cause vascular toxicity and impair normal tissue function. On the other hand, a previous study revealed that ketamine possessed significant anti-angiogenic effects, where 2.5 mg/mL KET markedly inhibited vascular network formation in the chick CAM model, indicating a concentration-dependent anti-angiogenic activity of KET (38). This discrepancy suggests that KET might modulate angiogenesis-related pathways only at higher concentrations, while retaining anticancer activity without directly affecting angiogenesis at low doses. Notably, upon testing at low concentrations, the KET+DDM combination significantly reduced MDA-MB-435 cancer cell viability while remaining safe for normal fibroblasts, associated with enhanced apoptosis, G2/M cell-cycle arrest, inhibition of migration and colony formation, and regulation of apoptotic markers (↑BAX, ↓BCL-XL, P53, and Caspase 8) (8). Thus, the lack of antiangiogenic activity observed in the CAM model supports the notion that KET's anticancer effects are selective rather than broadly cytotoxic to normal endothelial processes.

While the anesthetic effects of KET were evaluated using the CAM model, its impact on early embryogenesis was not examined (39). Moreover, exploring the toxicity of KET, DDM, and their combination (KET+DDM) in the early stages of embryogenesis provides critical information regarding the potential drug administration and its safety profile. Embryos treated with KET alone or in combination with DDM exhibited high survival rates, with no significant morphological abnormalities observed over the five-day exposure period. Moreover, these findings were supported by qPCR analysis, where molecular assessment of key organs, including the heart, lungs, kidneys, and brain, showed no widespread significant alterations in the expression of apoptotic genes (Caspase 3, Caspase 8, Caspase 9, and BAX) or the

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angiogenic marker VEGF. However, isolated significant differences were observed, including changes in Caspase 3 expression in the DDM-treated group and BAX expression in the KET-treated group compared to controls. It should be noted, however, that these molecular markers represent a limited panel and do not comprehensively capture all apoptotic or stress-related pathways. The VEGFR family plays a central role in regulating angiogenesis, making it a key therapeutic target for the development of anti-angiogenic agents (40,41). In contrast, treatment with DDM alone resulted in a noticeable reduction in embryo viability. It should be noted that qPCR was performed only on surviving embryos, so early or severe DDM-induced effects might have been missed. Moreover, rapid, non-apoptotic mechanisms, like membrane disruption, might induce toxicity without altering apoptotic gene expression. While DDM is widely regarded as a relatively safe non-ionic surfactant and has been used extensively to enhance drug delivery across biological barriers (42,43), its biological effects might be concentration- and exposure-dependent. Furthermore, the observed embryotoxicity of DDM alone might be attributed to membrane-disruptive properties associated with surfactants, particularly in rapidly developing embryonic tissues that are highly sensitive to changes in membrane integrity (44). However, this adverse effect was not observed when DDM was combined with KET, an observation that warrants further investigation to determine whether mechanistic interactions are involved. One possible explanation is that KET might exert a protective effect against DDM-induced embryotoxicity, potentially through modulation of stress-response pathways or membrane stabilization. These findings indicate that no significant changes were detected in the selected apoptotic and morphological parameters of angiogenesis-related markers during early embryogenesis, consistent with the observed embryonic tolerability of KET and its combination with DDM. Using the zebrafish model, the effect of KET on embryogenesis has also been evaluated, revealing developmental stage–dependent toxicity at early exposure, induced mortality, and skeletal malformations in a concentration-

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dependent manner, while low concentrations remained safe (45). In another work, high concentrations of KET also resulted in an induced dose-dependent mortality and growth defects, with 1820 µM of KET causing cardiac enlargement, structural malformations, and increased cardiac cell proliferation (46). Functional cardiac impairment was also evident through reduced heart rate, decreased ventricular shortening fraction, and downregulation of XMLC2 expression across multiple developmental stages (46). It was also reported that KET exposure in chick embryos suppressed spontaneous motility in a developmental stage–dependent manner, with acute treatment inhibiting movement from day 15 of incubation and chronic exposure impairing normal motility maturation, while short-term exposure to KET had limited effects at earlier embryonic stages (47).

To further support the safety of KET, DDM, and KET+DDM combination, a cytocompatibility assay was conducted using primary EFBs. Interestingly, the findings illustrated that neither KET nor DDM, alone or in combination, induced a significant reduction in cell viability or caused detectable morphological alterations following 48 h. of exposure. Primary EFBs are considered a physiologically relevant normal cell model, and the preservation of their viability and morphology indicates that the treatment groups did not exert nonspecific cytotoxic effects on healthy cells. This is particularly relevant when contrasted with our previous observations of reduced melanoma cell viability under similar treatment conditions, suggesting a degree of selectivity toward malignant cells (8).

It is important to highlight that some limitations of this work should be acknowledged. The CAM model, while highly informative for early-stage toxicity and angiogenesis assessment, does not fully recapitulate the complexity of mammalian pharmacokinetics or long-term systemic exposure, and thus our data needs further validation using alternative models to fully elucidate KET's impact on angiogenesis and early embryogenesis and to address evolutionary differences between species. Furthermore, this study

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analyzed only surviving embryos, thus early or severe DDM-induced toxicity might have been missing. Consequently, changes in apoptosis- and angiogenesis-related genes could be underestimated. Future work with earlier time points, longitudinal sampling, and additional molecular endpoints are needed to fully assess treatment-related effects. Overall, the findings of this study demonstrate that the KET+DDM combination did not produce measurable adverse effects in a normal developmental and cellular context, despite its previously reported anticancer activity. The lack of detectable effects on the tested morphological parameters of angiogenesis, embryotoxicity, or cytotoxicity in normal cells provides preliminary evidence that this formulation is compatible with the tested models under the conditions studied.

5. Conclusion and future directions

In conclusion, this study provides preliminary observations on embryonic, angiogenic, and cellular responses to KET and its combination with DDM using a chicken embryo platform. The study outcomes demonstrated that KET, DDM, and their combination (KET+DDM) showed no detectable adverse effects in both embryonic and cellular models, with no detectable morphological parameters of angiogenesis, embryonic development, or primary fibroblast viability under the tested conditions. The CAM model revealed preserved vascular morphology following treatment, while molecular analyses showed no widespread significant alterations in the selected apoptotic and angiogenesis-related genes. These findings indicate that the KET+DDM combination did not reduce survival under the tested conditions and suggest its potential as a selective anticancer strategy, while noting that broader safety effects on normal tissues remain to be evaluated. Importantly, the observed DDM-associated embryotoxicity when combined with KET suggests a protective or modulatory interaction that warrants further mechanistic investigation. Future studies should focus on dose-range and long-term safety evaluation in mammalian models to better understand

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systemic toxicity and pharmacokinetics, alongside mechanistic investigations into the molecular interactions between KET and DDM to elucidate the basis of enhanced therapeutic selectivity and potential interactions between KET and DDM. Overall, these findings provide preliminary evidence supporting the continued development of KET+DDM as a potential anticancer formulation, while emphasizing that further studies are needed to evaluate its safety and efficacy for future translational applications.

Statements and Declarations

Availability of data and materials

Data is provided within the manuscript.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Conflict of interest

Authors declared that there is no conflict of interest.

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Authors' Contributions

S.I.: Data curation, Formal analysis, Writing—original draft. **H.K.:** Data curation, Formal analysis. **A.H.F.:** Data curation, Formal analysis. **L.A.:** Supervision, Writing—review and editing. **K.A.A.:** Supervision, Writing—review and editing. **H.A.:** Supervision, Writing—review and editing. **A.E.:** Supervision, Writing—review and editing. **O.R.**: Supervision, Writing—review and editing. **A.M.A.:** Conceptualization, Main supervision, Validation, Funding acquisition, Methodology, Project administration, Resources,

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Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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