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Study of the effect of cow's milk on the vitality of protocolizes of the parasite Echinococcus granuloses in vitro

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Abstract
Cystic echinococcosis (CE) is a zoonotic infection caused by Echinococcus granulosus larval stage, with surgical intervention being the primary treatment, often accompanied by chemotherapy. Nevertheless, existing therapies such as benzimidazole carbamates and scolicidal agents may not always yield desired results. Recently, bovine lacteal fluid was tested on CE-causing Echinococcus granulosus protoscoleces in a laboratory. Protoscoleces were treated with cow's milk at doses of 4, 8, 12, 16, and 20 µM in vitro. The viability and structural alterations of a substance were evaluated using scanning electron microscopy(SEM) and transmission electron microscopy(TEM), and caspase-3 activity was measured using a colorimetric assay. The investigation revealed significant protoscolicidal effects when added to cow's milk at doses of 16 µM and 20 µM. After incubing them for 6 days, protoscoleces mortality rates reached 83.24% at 16 µM and 100% at 20 µM. According to SEM study, damage caused by drugs mostly came from the tegument. With more lipid droplets and vacuoles seen after 16 µM cow's milk exposure, TEM revealed serious interior tissue injury. After 24 hours of cow's milk incubation, protoscoleces had considerably higher caspase-3 activity than untreated samples. The current investigation displays the significant in vitro scolicidal impact of cow's milk against E. granulosus protoscoleces. However, further research is required to evaluate its effectiveness in vivo, clarify the precise mechanism of action, and determine any potential side effects.

Keywords: Cow's milk, Protoscolices, Echinococcus

1. Introduction

The tapeworm Echinococcus granulosus larvae infect humans, causing hydatid disease or cystic echinococcosis (CE) (Díaz, 2017; Gessese, 2020; Santucciu et al., 2020). Direct contact with infected animals is the main vector for the transmission of this zoonotic disease to humans (Rahman et al., 2020; Nicoletti, 2020; Qiu et al., 2023; de Silva et al., 2023). CE primarily affects the liver and lungs, resulting in space-occupying lesions and severe complications (Naar et al., 2020; Coyle and Junghanss, 2020). About 75% of CE cases involve hepatic cysts, while approximately 15% involve pulmonary cysts (Wu et al., 2021; Santucciu et al., 2023; Kaya et al., 2023). CE is prevalent in developing regions where livestock is crucial to the local economy (Avila-Granados et al., 2019).

Treatment strategies for cystic echinococcosis (CE) are complex and primarily depend on the specific characteristics of the cyst (Velasco-Tirado et al., 2018; Budke et al., 2017; Rossi et al., 2020). The main therapeutic approach currently involves surgically removing the parasitic mass, particularly in cases with complicated cysts (Thapa et al., 2018; Gavara et al., 2015). Puncture, Aspiration, Injection, and Re-aspiration (PAIR) is an increasingly common minimally invasive procedure, particularly ideal for patients who are ineligible for surgery or who decline it. Although surgery often results in full recovery, it does not completely prevent recurrence (Filippou et al., 2007). Accidental protoscolece spillage during surgery and failure to remove dead space can significantly contribute to recurrence and the development of multiple secondary CE cases. Studies have shown a high recurrence rate associated with surgery (Wen et al., 2019).

Chemotherapy, involving powerful drugs, has been crucially utilized as an adjunct to surgical treatments (Sugarbaker and Van der Speeten, 2016). The localized application of chemotherapeutic agents during surgery can significantly reduce the risk of viable protoscolex spillage and subsequent infection in the surrounding tissue (C. Elissondo et al., 2013). Hypertonic saline and medicinal

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alcohol may harm liver tissue during surgery and induce postoperative hepatobiliary problems, therefore innovative, safe, and efficient scolicidal medicines are needed (Kohansal et al., 2017).

A study was conducted to evaluate the in vitro effects of cow's milk as a potential scolicidal agent on Echinococcus granulosus protoscoleces growth, morphology, and ultrastructure. An early experiment was conducted to better understand the complex mechanism of action of cow's milk on protoscoleces. We strongly believe that uncovering the unique properties of cow's milk will significantly advance the development of innovative treatment approaches aimed at preventing the recurrence of cystic echinococcosis (CE) (Wang et al., 2018).

2. Material and Method

2.1. The in vitro cultivation of Echinococcus granulosus protoscoleces was executed through the following procedure:

Hydatid cysts were aseptically perforated from sheep livers that had become infected on their own at the Manasi slaughterhouse in Xinjiang, China, to get protoscoleces. The protoscoleces were washed 3-5 times with PBS (pH 7.2) to remove membrane debris and non-viable protoscoleces. The parasites' viability was evaluated using a 0.1% eosin staining test, and only batches with over 98% viability were chosen for further experimentation.

Protoscoleces were obtained from sheep livers in Xinjiang, China, through puncturing hydatid cysts. Following the collection of the samples, they were washed three to five times with PBS (pH 7.2) in order to dispose of membrane debris and non-viable protoscoleces. To determine viability, a 0.1% eosin staining test was used. Only batches that showed viability levels over 98% were chosen for further testing.

These viable protoscoleces were then cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), phenol red, and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). The cultures were maintained at 37°C with 5% CO2, with the medium refreshed every 3-5 days to ensure optimal growth conditions. This in vitro culture setup aimed to provide a controlled environment conducive to studying the growth and characteristics of Echinococcus granulosus protoscoleces for further experimental observations.

2.2. Cow's milk Inhibition Studies

To explore the inhibitory effects of cow's milk, experiments were conducted using protoscoleces of Echinococcus granulosus. Each well of round-bottom 6-well plates was seeded with approximately 3,000 protoscoleces. Different concentrations of cow's milk (4, 8, 12, 16, 20 µM) and control components were employed for the investigations.

Protoscoleces were cultured in a humidified incubator set at 37°C with 5% CO2 using an identical amount of RPMI 1640 culture solution for cow's milk controls. The incubation conditions were carefully monitored to ensure optimal conditions for the protoscoleces. To maintain consistent treatment conditions, repeat treatments were administered every 3 days.

Each culture, including 5 ml of incubation media, was kept in a humidified incubator set at 37°C with 5% carbon dioxide. Protoscoleces were incubated with RPMI 1640 culture medium in equivalent amounts for cow's milk controls. The incubation conditions were carefully monitored to ensure optimal conditions for the protoscoleces. To maintain consistent treatment conditions, repeat treatments were administered every 3 days.

2.3. Morphology and Viability Analysis of Protoscoleces

To comprehensively evaluate the impact of daily dosing with various concentrations of cow's milk, daily samples of protoscoleces were collected from each dosing group. The study assessed morphology and viability of 80-100 protoscoleces in 150 µl of incubation medium using a 0.1% eosin staining test following a established protocol. This work assessed the vitality of protoscoleces by calculating the proportion of viable protoscoleces out of the total number seen in 10 fields that were

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chosen at random. This evaluation was conducted using phase-contrast microscopy to ensure precise and detailed analysis.

To ensure reliability and consistency, each viability test was performed with three replicates per treatment condition. Additionally, the entire experiment, involving daily sampling and viability assessment, was rigorously repeated three times. This meticulous approach aimed to provide a thorough understanding of how different concentrations of cow's milk influence both the morphology and viability of Echinococcus granulosus protoscoleces over consecutive days. It offered valuable insights into the effectiveness and potential dynamic changes induced by the treatment.

2.4. Protoscoleces Ultrastructure Observation

The research obtained protoscolece samples by subjecting them to incubation with cow's milk at doses of 8 µM and 16 µM for durations of 3 and 5 days, using standard procedures as previously outlined by Cumino AC and Elissondo M. Subsequently, the protoscoleces incubated with cow's milk were prepared for ultrastructural observation.

For SEM analysis, samples were meticulously prepared and observed using a LE01430VP scanning electron microscope operated at 20 kV. This method enabled detailed examination of the surface morphology of the protoscoleces. Ultrathin sections, ranging from 80 to 100 nm in thickness, were obtained using an LKB2088V ultramicrotome equipped with a diamond knife. These sections were then stained with uranyl acetate and lead citrate.

For TEM analysis, a JEOL1230 transmission electron microscope operating at 80 kV was utilized. TEM provided a comprehensive view of internal cellular structures at the subcellular level.

2.5. Caspase-3 Activity Colorimetric Assay

Following particular treatments, the caspase-3 enzyme activity in protoscoleces was assessed using a caspase-3 test kit as directed by the manufacturer. Cytoplasmic proteins (3 mg) from cow's milk-treated protoscoleces (4, 8, 12, 16, and 20 µM) were combined with reaction buffer and caspase-specific substrates (Ac-DEVD-ρNA for caspase-3) for 24 hours. After dispensing this mixture onto 96-well plates, it was incubated at 37°C for 4-6 hours.

After incubation, ρNA absorbance at 405 nm was measured using a Bio-RAD microplate reader (USA). Sample-specific absorbance values were obtained by subtracting the background absorbance of the negative control (assay buffer substrate). To make sure the study of caspase-3 activity in reaction to the different amounts of cow's milk was accurate, the mean values from three measures were found.

2.6. Statistical Analysis

The experiments were meticulously conducted in triplicate, and the resulting data were reported as means ± standard deviations (SDs) to accurately represent variability. Statistical analyses, including t-tests, One-Way ANOVA, or LSD (Least Significant Difference), were performed using SPSS 17.0 software.

A significance level of less than 0.05 ($P < 0.05$) was considered statistically significant, providing a robust framework for evaluating the impact of cow's milk concentrations on various parameters. This approach ensured the reliability and validity of the observed effects and allowed for informed conclusions regarding the inhibitory actions and changes induced by the treatment on Echinococcus granulosus protoscoleces

3. Results

3.1. The morphology of E. granulosus Protoscoleces: in vitro effect of cow's milk

The study revealed that Echinococcus granulosus protoscoleces remained structurally consistent under an inverted microscope(Fig. 1A-B), but exposure to cow's milk caused significant morphological changes, including eosin absorption and a distinctive red coloration. Deceased protoscoleces displayed eosin absorption, resulting in a distinctive red coloration. After 4 days of incubation with 16 µM cow's milk, evaginated-type protoscoleces displayed disorganization, partial

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hook desquamation, and sticky disc deformation, leading to lower activity and constriction of the soma area (Fig. 1E).

Over time, the number of deceased protoscoleces increased, with numerous blebs observed by day 8 and a reduction in calcareous corpuscles under an inverted microscope (Fig. 1F). Similar changes were also seen between 4 and 8 days post-incubation with 8 µM cow's milk (Fig. 1C-D). The study found that cow's milk's effects on protoscoleces were concentration-dependent and time-dependent, with higher concentrations and longer incubation periods causing more lethal effects. After 8 days, no viable protoscoleces were detected.

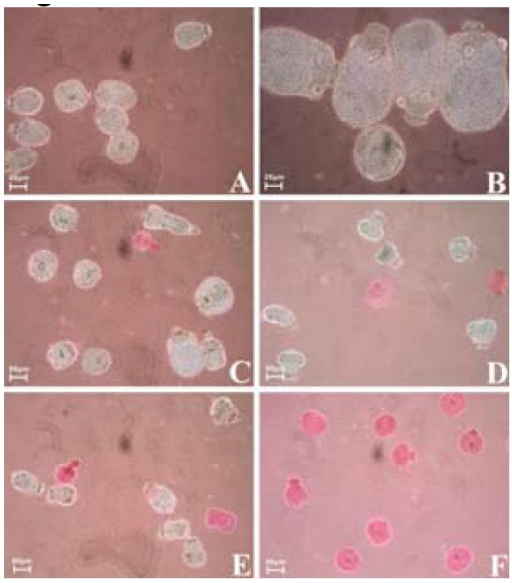

Fig 1. The protoscoleces of Echinococcus granulosus were subjected to treatment with Cow's milk and control compounds, and their morphological changes were observed under a light microscope. The descriptions for each image are as follows: (A) Control protoscoleces at 100× magnification. (B) Control protoscoleces at 400× magnification. (C) Protoscoleces showing alterations after 4 days' post-infection (p.i.) with 8 µM Cow's milk at 100× magnification. (D) Protoscoleces displaying alterations after 8 days p.i. with 8 µM Cow's milk at 100× magnification. (E) Protoscoleces exhibiting changes after 4 days p.i. with 16 µM Cow's milk at 100× magnification. (F) Protoscoleces with modifications after 8 days p.i. with 16 µM Cow's milk at 100× magnification.

3.2. the Viability of E. granulosus Protoscoleces: In Vitro Effect of cow's milk

Cow's milk affects Echinococcus granulosus protoscoleces vitality (Figure 2). The vitality of control protoscoleces was similar throughout the investigation. Viability dropped to 93.5% by day 9. The protoscolicidal impact of cow's milk at varying concentrations was evident, particularly in the highest concentration group (≥12 µM), which demonstrated a substantial growth inhibition (Fig. 2).

This figure provides a clear visualization of the declining viability trend in control protoscoleces and emphasizes the protoscolicidal effects induced by different concentrations of cow's milk, especially at higher levels. It underscores the inhibitory impact of cow's milk on Echinococcus granulosus protoscoleces.

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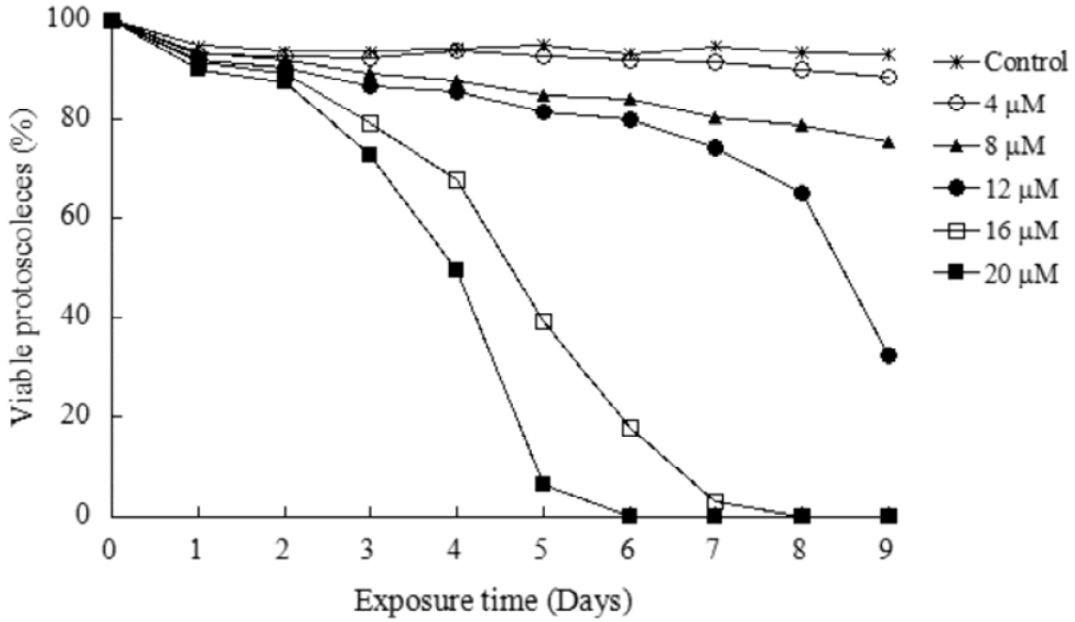

Fig 2. The viability loss of Echinococcus granulosus protoscoleces was evaluated during in vitro treatment with cow's milk. The mean percentage of essential protoscoleces from three distinct investigations is represented by each data point. It is important to note that the effects of cow's milk are both time- and dose-dependent.

The study revealed that prolonged exposure to 20 μM cow's milk significantly impacted protoscolicidal activity, with no viable protoscoleces observed after 6 days. In the same vein, the inhibitory effect was significantly enhanced in comparison to 12 μM cow's milk, as mortality reached 100% at concentrations of 16 μM by day 8. As opposed to the higher concentration groups, the protoscolicidal impact was less noticeable in the low concentration group (≤8 μM).

Our findings demonstrate that, after eight days of incubation, protoscoleces cultured in 4 μM or 8 μM cow's milk were still viable, with viability percentages of 90.23% and 78.71%, respectively. This demonstrates how cow's milk's scolicidal action against Echinococcus granulosus protoscoleces is dose- and time-dependent.

Ultrastructure of E. granulosus Protoscoleces: In Vitro Effect of Cow's milk

Protoscoleces were prepared for SEM and TEM studies to evaluate structural changes caused by cow's milk exposure, and control cultures showed no noticeable ultrastructural changes in parasite tissue throughout the incubation period (Figs. 3A, 3B, and 4A). In contrast, protoscoleces treated with cow's milk exhibited evident morphological and ultrastructural damage, as observed in both SEM and TEM analyses.

The parasite tegument was identified as the fundamental site of drug-induced damage, with significant ultrastructural changes seen by day 3 post-incubation with cow's milk, including protoscolex evagination, slight soma contraction, and rostellar deformation (Fig. 3C). This stage saw no changes to the microtriches. On day 5, the soma area contracted, the sticky disc deformed, hooks and rostellum disorganized, and microtriches partially shed (Fig. 3D). Furthermore, at 16 μM cow's milk, these signs of ultrastructural changes with markedly altered tegument were even more pronounced.

These observations underscore the significant impact of cow's milk on the ultrastructure of Echinococcus granulosus protoscoleces, particularly targeting the tegument, as evidenced by SEM and TEM analyses.

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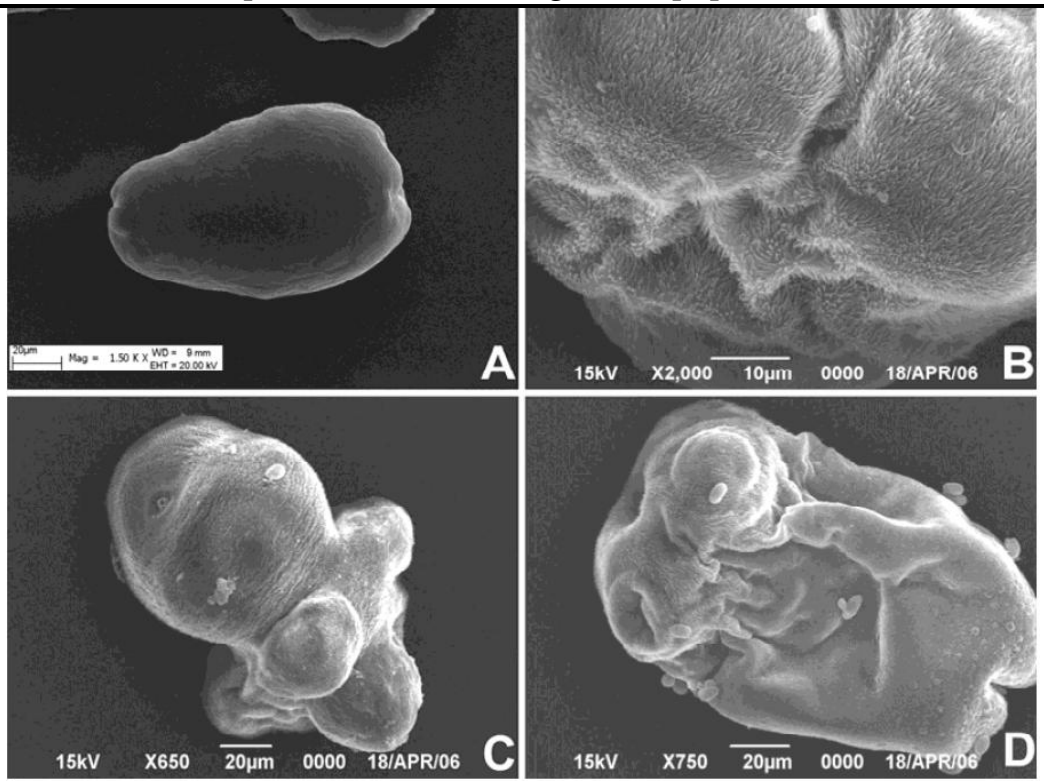

Fig 3. To investigate Echinococcus granulosus protoscoleces cultured in vitro with or without cow's milk, scanning electron microscopy was used. The following are the descriptions for every image: (A) Protoscoleces grown in culture solution RPM 1640. (B) Scolex region of an evaginated control protoscolex. (C) Protoscoleces exhibiting alterations after 3 days' post-incubation (p.i.) with 8 µM Cow's milk. Notably, the soma region has undergone contraction. (D) Protoscoleces displaying modifications after 5 days p.i. with 8 µM Cow's milk. The soma region appears severely contracted in this instance.

Upon TEM examination of protoscoleces treated with the drug, it was observed that 8 µM cow's milk resulted in a thinner glycocalyx and shedding of microtriches (Fig. 4A-B), accompanied by the appearance of small vacuoles in the distal cytoplasm (Fig. 4C). These effects were notably more pronounced with 16 µM cow's milk compared to 8 µM, as evidenced by more substantial changes in the ultrastructure (Fig. 4D-F).

There were significant alterations in the ultrastructure three days after incubation, including the presence of large vacuoles in the distal cytoplasm and vacuolation of the proximal cytoplasm (Fig. 4D). TEM studies also showed the breakdown and fragmentation of the syncytium, which greatly deteriorated the interior tissues and caused integrity loss. Along with this, the count of vacuoles and lipid droplets increased (Fig. 4E). Moreover, the lamellar residual bodies in several protoscoleces increased significantly (Fig. 4F).

These TEM observations provide a detailed insight into the complex ultrastructural changes induced by cow's milk, highlighting the dose-dependent impacts on various cellular components of Echinococcus granulosus protoscoleces.

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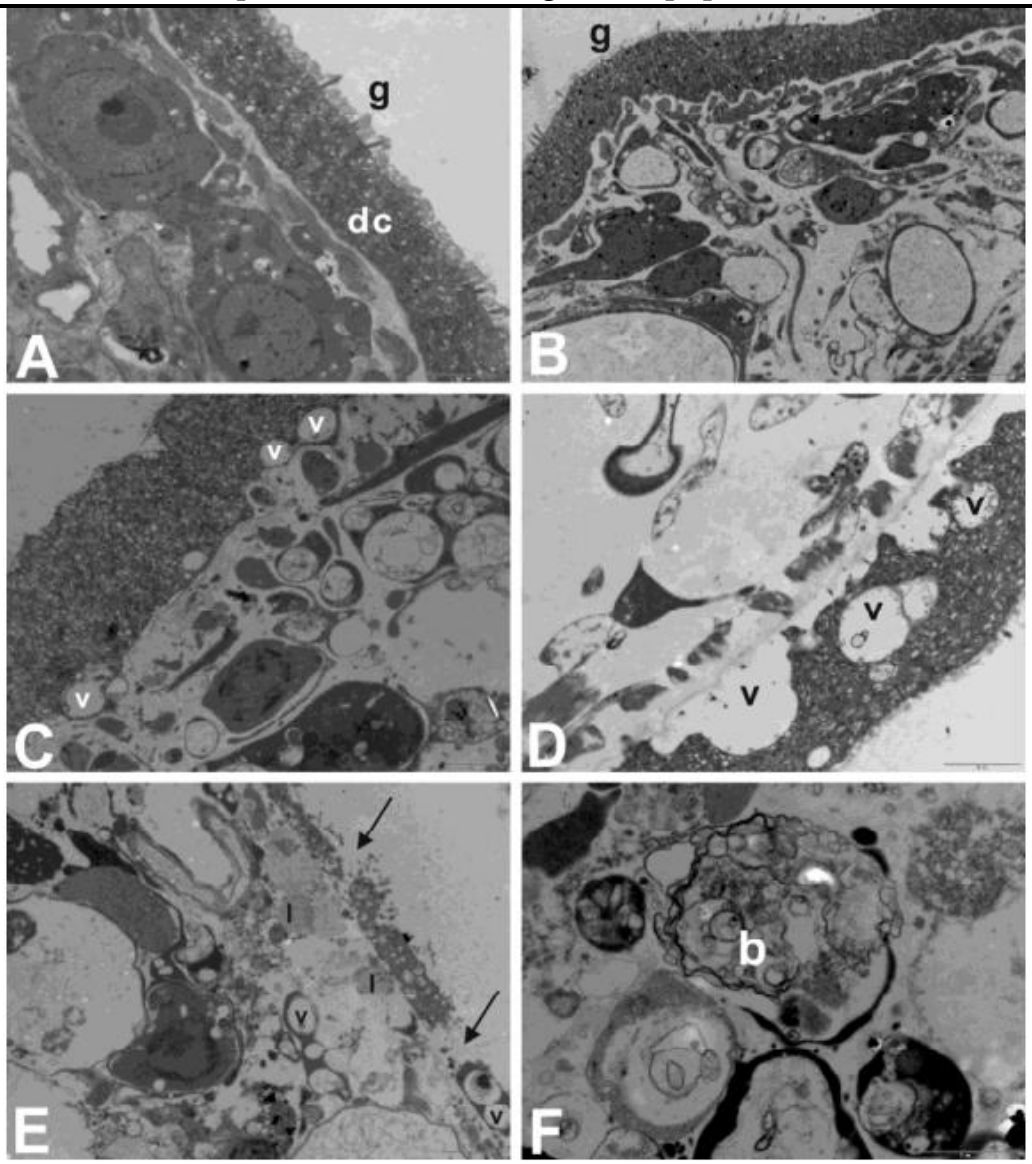

Fig 4. Transmission electron microscopy was utilized to investigate Echinococcus granulosus protoscoleces incubated in vitro with Cow's milk. Descriptions for each image are as follows: (A) Soma region of a control protoscolece (g glycocalyx, dc distal cytoplasm; ×10,000). (B) Treated protoscolex (3 days post-incubation, 8 µM Cow's milk). Notable features include a thinner glycocalyx and truncated microtriches (g; ×6,000). (C) Treated protoscoleces (5 days p.i., 8 µM Cow's milk). Small vacuoles (v) are observed in the distal cytoplasm (×8,000). (D) Protoscoleces incubated with 16 µM Cow's milk (3 days p.i.). Internal tissues are significantly impacted, characterized by the presence of large vacuoles (v) in the distal cytoplasm and vacuolation of the proximal cytoplasm (×10,000). (E-F) Protoscoleces were incubated with 16 µM Cow's milk for 5 days post-infection. (E) The syncytium is fractured and disintegrated (arrow). There are 8,000 lipid droplets (l) and vacuoles (v). (F) Residual lamellar bodies (b; ×15,000).

Apoptosis Induced by Caspase-3 in Cow's Milk in Vitro

The start of apoptosis requires the activation of caspase proteases. Caspase-3 activity was measured in protoscoleces treated with cow's milk after 24 hours at varied concentrations employing a colorimetric assay with AcDEVD-ρNA as a substrate to evaluate apoptotic cell death (Table 1). In comparison to control protoscoleces, the investigation revealed a concentration-dependent pattern of a substantial increase in caspase-3 activity in protoscoleces treated with cow's milk for 24 hours (P <

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0.05) (Table 1). These results clearly imply that cow's milk-induced death in protoscoleces might be caspase-3-dependent mechanism.

Table 1. The impact of Cow's milk on caspase-3 activity in E. granulosus protoscoleces was assessed after a 24-hour exposure (Relative Optical Density, x ± S, n=3).

Group	Caspase-3 relative activity
Control	1±0.0000
4 μM Cow's milk	1.4455±0.0201*
8 μM Cow's milk	1.5154±0.0362*
12 μM Cow's milk	1.6671±0.0371*
16 μM Cow's milk	1.9281±0.0406*
20 μM Cow's milk	2.3337±0.0766*
*P<0.05 compared with the control group.	

4. Discussion

Scolicidal drugs must be used during hepatic hydatid cyst surgery to avoid protoscoleces leakage and infection. Despite the potential adverse effects, the contents of hydatid cysts have been sterilized using a variety of scolicidal substances, including hypertonic saline (20%), ethanol (95%), H2O2, and albendazole.

In modern medical practices, cow's milk has demonstrated significant efficacy in treating various cancers, particularly Acute Promyelocytic Leukemia (APL), due to its ability to induce apoptosis in leukemia cells. Furthermore, substances obtained from cow's milk are crucial to the production of agricultural necessities such as fungicides, insecticides, and drugs for the eradication of tapeworms in cattle. Medications generated from cow's milk are used in the field of veterinary medicine for the purpose of treating parasite diseases. These diseases include amoebic dysentery, filariasis in dogs, and blackhead in fowl and turkeys.

Cow's milk has also shown promise in combating African trypanosomiasis, exhibiting significant antileishmanial activity. Recent work by Antimisiaris et al. shows, after 24 hours of incubation, the in vitro anti-trypanosomal efficiency of arsonoliposome formulations. These studies underscore cow's milk's accumulation in the mouse liver at high concentrations, aiding anti-protozoal therapy for visceral leishmaniasis, where parasites predominantly inhabit the liver. However, there remains a significant research gap in understanding the effects of cow's milk on Echinococcus granulosus protoscoleces.

The in vitro protoscolicidal impact of cow's milk on Echinococcus granulosus protoscoleces is investigated in this work, with increasing milk content and exposure period results in increasing treatment efficacy. Cow's milk exposure at concentrations of 4 μM and 8 μM led to a gradual decline in viable protoscoleces, with viabilities of 91.78% and 83.98% after 6 days. The viability of protoscoleces decreased significantly after exposure to 16 μM cow's milk, reaching 16.76% after 6 days, and completely dying after 8 days, while 20 μM cow's milk led to 100% mortality within 6 days.

5. Conclusion

The viability assay results align with the observed structural tissue damage, confirming that incubation with cow's milk induces significant ultrastructural alterations, primarily affecting the tegument of the protoscoleces. The main area of injury seems to be the tegument, which exhibits significant alterations.

Ultrastructural changes in the scolex include deformation of the adhesive disc, contracting soma area, rostellar disarray, loss of hooks, and scattering of microtriches. The digitiform tegumental projections and uneven holes in protoscoleces grown with higher cow's milk amounts are particularly intriguing.

Transmission Electron Microscopy (TEM) studies show that protoscoleces have more ultrastructural affects on the inside. The vacuolization of the distant cytoplasm is becoming more apparent, and the

germinal layer contains lipid droplets or lamellar remnant bodies. These changes in internal tissue have very bad effects, and the amount of damage is directly linked to the dose of the drug that was tried.

Treatment with 16 μM cow's milk induces significant ultrastructural and degenerative changes, particularly impacting the destruction of the syncytium and leading to widespread cell demise within the germinal layer. Caspase-3 emerges as pivotal in orchestrating apoptosis through various metabolic pathways. Our findings consistently show increased caspase-3 activity in protoscoleces exposed to cow's milk, underscoring its role in apoptosis activation.

While the exact molecular mechanisms underlying cow's milk's antiparasitic actions remain unclear, our results reveal a substantial rise in caspase-3 activity across varying concentrations of cow's milk compared to controls. Notably, protoscoleces exposed to 20 μM cow's milk exhibit peak caspase-3 activity at 2.3337 ± 0.0766, highlighting a dose-dependent response.

These in vitro findings suggest cow's milk holds promise as a scolicidal agent, demonstrating efficacy in deactivating hydatid cysts during surgical interventions. Further comprehensive studies should explore cow's milk's protoscolicidal activity, particularly focusing on shorter exposure times. Such investigations will deepen our understanding of cow's milk's scolicidal properties and potentially optimize its application in managing hydatid cysts during surgical procedures.

In conclusion, our study underscores cow's milk's potent scolicidal activity against Echinococcus granulosus protoscoleces. Future steps involve evaluating its safety and efficacy in animal models and elucidating its molecular mechanisms. This effort promises insights into cow's milk's targets and advancements in treatments for human cystic echinococcosis.

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