

Effect of the use of acetylcysteine on the reduction of the percentage of neutrophil polymorphonuclear cells present in subclinical endometritis of dairy cattle diagnosed with the cytobrush technique

Efecto del uso de la acetilcisteína sobre la reducción del porcentaje de polimorfonucleares neutrofilos presentes en la endometritis subclínica de ganado vacuno de leche diagnosticada con la técnica de citobrush

FERNANDEZ FERNANDEZ, F^{1,2,5}; PACHECO, K¹; HASAN ICEN³; SERVET BADENKYRAN³; PACHECO, V²; LOMBARDO, DM^{4,6}

¹Universidad Católica de Santa María, Arequipa, Perú. ²Vicerrectorado de Investigación. Arequipa, Perú. ³Dicle University- Diyarbakir – Turquía. ⁴Universidad de Buenos Aires. Facultad de Ciencias Veterinarias. Instituto de Investigación y Tecnología en Reproducción Animal (INITRA) Argentina. ⁵Escuela de Postgrado Universidad Católica de Santa María. Arequipa, Perú. ⁶Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

ABSTRACT

The objective of the present study was to evaluate the effect of N-acetylcysteine (NAC) administration as a pretreatment prior to antibiotic therapy in the control of subclinical endometritis (SE). Endometrial mucosa samples were collected from 14 housed Holstein Friesian cows from the Majes Irrigation Area, Caylloma, Arequipa, Peru. The animals were between their first and fourth lactation, with a body condition score (BCS) of 4. A cervical-uterine cytological brush was used for Papanicolaou sampling. Smears were prepared and submitted to the laboratory, stained using the Diff-Quick technique, and examined under bright-field microscopy for the count and calculation of the percentage of polymorphonuclear neutrophils (%PMN-N). SE was confirmed when %PMN-N was $\geq 5\%$. The brushes were transported in tubes containing Amies transport medium for subsequent aerobic culture on MacConkey agar, Mueller–Hinton agar, blood agar base, and Chromagar, with bacterial identification from the endometrial mucosa performed through Gram staining and biochemical tests. Antibigrams were carried out according to the bacteria identified. Of the 14 cows sampled, 11 showed %PMN-N values above the 5% threshold and were classified as SE-positive, with those below this value excluded from further analysis. Two groups were formed: T-NAC (n = 5) and nT-NAC (n = 5). The T-NAC group received an intrauterine infusion of 40 cm³ of 2% NAC (Fluimucil® 300 mg/3 mL) for three consecutive days, starting 72 hours after sampling, while the nT-NAC group served as a control (no NAC treatment). Endometrial bacteria were isolated in four cows, identified as *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Escherichia coli*. Twelve hours after NAC administration, differentiated intrauterine antibiotic treatment was applied to both groups according to the bacterial species identified. Seven days after the first sampling, vaginal cytology was repeated to reassess %PMN-N. Data were analyzed using InfoStat 2020 software. Summary and dispersion measures were calculated; for the total animals evaluated for PMN-N (n = 14), the median was 9.50 with a standard error (SE) of 1.21. Paired-sample inference was performed using the Student's t-test for both treatment and control groups with a two-tailed contrast, yielding a statistically significant effect for the T-NAC group (p < 0.05) and a non-significant result for the nT-NAC group (p > 0.05). In conclusion, intrauterine administration of N-acetylcysteine in cows with subclinical endometritis significantly reduces %PMN-N.

Keywords: (cows), (subclinical endometritis), (biofilms), (N-acetylcysteine)

RESUMEN

El objetivo del presente estudio fue evaluar el efecto del uso de N- acetilcisteína (NAC) como tratamiento previo a la antibioticoterapia durante el control de endometritis subclínica (ES). Se obtuvieron muestras de la mucosa endometrial de 14 vacas Holstein Fresian, estabuladas, de la Irrigación Majes, Caylloma en Arequipa, Perú. Los animales se encontraban entre primera y cuarta lactancia, con una condición corporal de 4. Se utilizó una brocha ginecológica citológica cérvico uterina para Papanicolau. Se realizaron frotices y se remitieron a laboratorio. Se colorearon con la tinción de Diff Quick, observándose bajo microscopia de campo claro para el conteo y cálculo del porcentaje de polimorfonucleares neutrófilos (%PMN-N), confirmando la ES cuando el % PMN-N fue $\geq 5\%$. Se transportaron las brochas en tubos de transporte con medio Amies para su posterior cultivo aerobio en medios: MacConckey, Mueller Hinton, Agar base Sangre y Chromagar, e identificación de bacterias en mucosa endometrial mediante Tinción de Gram y bioquímica. Se realizaron los antibiogramas respectivos de acuerdo a la bacteria identificada. De las 14 vacas muestreadas, 11 presentaron un %PMN-N por encima del punto de corte del 5%, siendo positivas a ES, descartándose las de menor porcentajes. Se formaron dos grupos (T-NAC=5) y (nT-NAC=5), el grupo T-NAC recibió una infusión intrauterina de 40 cm³, por 3 días consecutivos de NAC al 2 % (Fluimucil ® 300mg/3 mL), 72 horas posteriores al muestreo, mientras que el nT-NAC sirvió de control (sin tratamiento de NAC). Se identificaron bacterias endometriales en 4 vacas, de las especies *Staphylococcus epidermidis*, *Staphylococcus aureus* y *Escherichia coli*, a las cuales 12 horas posteriores a la aplicación de NAC, se aplicó tratamiento antibiótico intrauterino diferenciado en ambos grupos. A los 7 días del primer muestreo se volvió a realizar la citología vaginal para evaluar %PMN-N. Los datos se analizaron usando el software InfoStat 2020. Se estudiaron las medidas de resumen y dispersión, obteniendo del total de animales evaluados para PMN-N (n=14), una mediana de 9.50 y EE (error estándar) de 1,21. Así mismo se realizó la inferencia para dos muestras apareadas mediante la prueba de T, para el grupo tratamiento y el grupo control con contraste bilateral, resultando para el grupo T-NAC el tratamiento significativo ($p < 0,05$) y para el grupo nT-NAC no significativo ($p > 0,05$). Se concluye que el uso de N-acetilcisteína, aplicada intrauterinamente en vacas con ES, disminuye el % PMN-N.

Palabras clave: (vacas), (endometritis subclínica), (biofilms), (N-acetilcisteína)

INTRODUCTION

After parturition, bovine endometrial epithelial cells (BEECs) undergo severe inflammation and an imbalance between oxidation and antioxidation, which is widely recognized as an important component in the development of endometritis in dairy cows. However, the mechanisms of inflammation and oxidative stress-mediated damage in BEECs remain inadequately defined, particularly the molecular pathways associated with mitochondria-dependent apoptosis¹².

One of the major achievements in reproductive management of dairy cows is achieving pregnancy as early as possible after calving (reducing the calving interval), as this results in more lactation periods and more calves over their productive lifetime¹⁹.

Although the bovine endometrium possesses numerous defense mechanisms that protect it from nonspecific invasive agents (pseudostratified epithelial cells, mucus secreted by endometrial

glands, and the action of polymorphonuclear cells and humoral antibodies), it is not exempt from invasion and damage by different microorganisms that can trigger subclinical infections^{6,7}.

Subclinical endometritis (SE), recently defined, may occur at any time following the histological completion of uterine involution (e.g., during or after week 8 postpartum), and is characterized by an endometrium extensively infiltrated with neutrophilic granulocytes, which can only be detected by cytological examination of the endometrium¹⁵. Either no or only minimal amounts of exudate accumulate in the uterus, resulting in a complete absence of pathognomonic cervical discharge^{3, 8, 10, 21}.

SE is a chronic, subclinical inflammatory process of the endometrium, with a relatively high proportion of PMN leukocytes in the uterus, which compromises fertility in affected cows¹⁷. The proportion of PMN cells

considered “relatively high” depends on the sampling technique as well as the postpartum interval^{10, 18}. In repeat breeder cows, suspicion is confirmed when the %PMN-N is $\geq 5\%$ ^{2, 16}.

N-acetylcysteine (NAC), a derivative of L-cysteine and a precursor of glutathione, acts to reduce mucus viscosity by breaking disulfide bonds linking proteins in mucoproteins, thereby eliminating purulent or non-purulent secretions from the body²⁰. NAC is used as a mucolytic treatment in obstructive pulmonary diseases, tuberculosis, and cystic fibrosis in human medicine. Beyond its mucolytic effect, NAC also exhibits antioxidant activity as a precursor of glutathione and by directly scavenging reactive oxygen species (ROS)^{4, 23, 25}.

Endometrial cytology has been employed as a diagnostic tool in equines, and a modified cytobrush has been used to collect endometrial cytology samples in cows without adversely affecting endometrial histology or epithelial architecture. When NAC infusion was included in treatment regimens prior to breeding, it was associated with higher pregnancy rates in subfertile mares⁹. Likewise, anti-inflammatory effects have been demonstrated in the uterine mucosa of mares²⁴. Repeat breeder mares, showing evident mucus hypersecretion but without uterine pathogens, achieved pregnancy rates of 77% when treated with NAC followed by uterine lavage post-mating and oxytocin (in some

cases combined with intrauterine antibiotics)^{5, 9}.

The use of NAC in clinical endometritis (CE) in cows resulted in a cure rate of 83.3% and pregnancy rates of 66.7%²³.

In light of these considerations, the aim of the present study was to evaluate the effect of N-acetylcysteine (NAC) as a pretreatment prior to antibiotic therapy in the management of subclinical endometritis (SE).

MATERIALS AND METHODS

Study site

This research was conducted in housed Holstein Friesian cows from the Majes Irrigation District, Caylloma Province, Arequipa Department, Peru, geographically located at 16°20'08.35" S latitude and 72°09'09.56" W longitude, at an average altitude of 1300 m above sea level. Laboratory analyses were carried out in the Research Laboratories of the Universidad Católica de Santa María, Pavilion F-403.

Animals and experimental design

A total of 14 Holstein Friesian cows between the 1st and 4th lactation, at 50–60 days postpartum, housed under intensive systems, with a body condition score (BCS) of 4, and clinically and reproductively healthy were included. Following endometrial cytology, 11 cows presented PMN-N $\geq 5\%$. The lowest value was excluded ($n = 10$), resulting in an explanatory-level study with two groups: Treatment (T-NAC = 5) and Control (nT-NAC = 5).

Table 1. Formation of experimental groups

GROUP	ID	Bacteria identified	%PMNs
T-NAC	1		8
	2	- <i>Staphylococcus epidermidis</i>	12
	3	- <i>Escherichia coli</i>	17
	4		9
	5		11
nT-NAC	8	- <i>Staphylococcus aureus</i>	16
	9	- <i>Staphylococcus epidermidis</i>	12
	10		8
	13		13
	14		10

Endometrial cytology (EC)

Endometrial cytology was used to determine SE, with certain limitations, also serving to evaluate uterine defense. The method consisted of obtaining cytological samples from the uterine lumen and assessing them by stained smears to determine the percentage of neutrophils¹⁰.

A cervical-uterine cytological brush for Papanicolaou sampling was used. The handle was cut 3–4 cm from the brush, which was then inserted into a 65 cm long, 4 mm diameter steel shaft, covered by a 50 cm long outer steel tube with an internal diameter of 5 mm, which protected the brush. The entire device was further covered by a sanitary sheath¹⁰.

During insertion and withdrawal from the genital tract, the brush remained protected inside the steel tube, being exposed only within the uterus to collect the sample. The procedure included cleansing of the perineal and vaginal area with sterile saline-moistened wipes, insertion of the brush into the uterine lumen, removal from the protective steel tube, and rotation against the wall of the largest horn. The brush was then re-sheathed and withdrawn. Smears were prepared on clean, degreased, sterilized glass slides (120 °C × 20 min). Diff-Quick staining was applied, and slides were examined under bright-field optical microscopy at ×400 magnification. Images were captured using Amscope MT1 software. A sample was considered positive according to Kasimanickam *et al.*¹⁰, Sheldon *et al.*²¹, and Arenas *et al.*² when %PMN-N ≥ 5%.

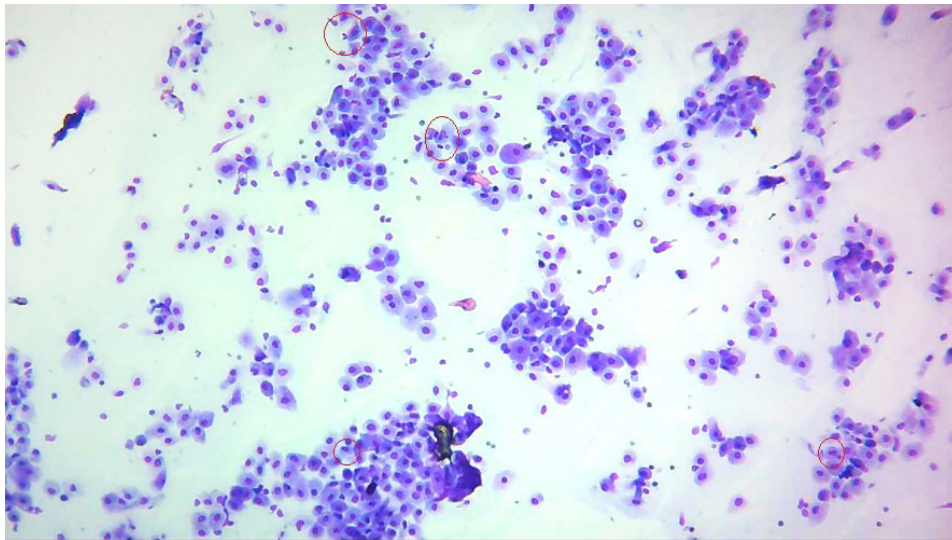


Figure 1. PMN Mucosal smear from the uterine lumen of cows x100

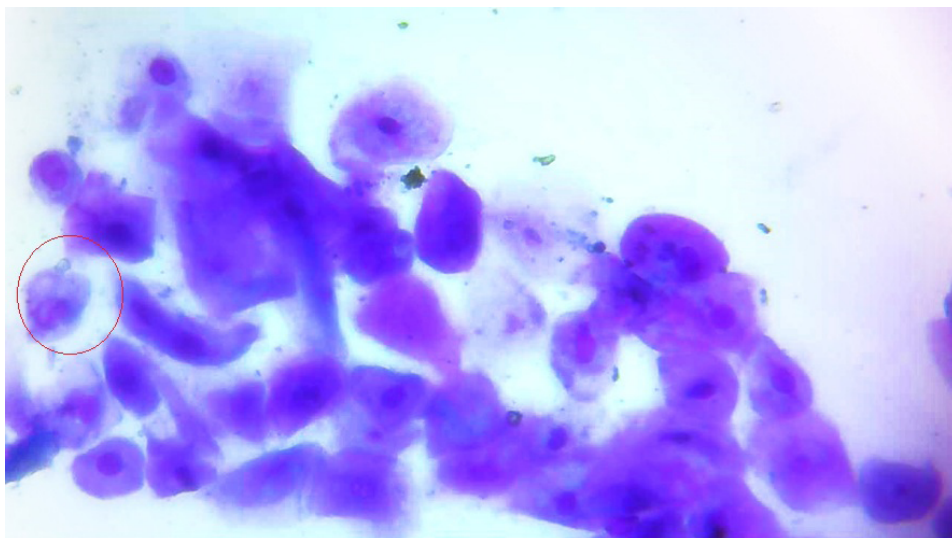


Figure 2. PMN in the mucosa of the uterine lumen of cows x400

Culture and antibiogram of endometrial cytology samples

Cytobrushes were transported in Amies transport medium tubes (Eurolab®, Germany) for subsequent aerobic culture by streaking onto Petri dishes. Media used included BBL™ MacConkey DB®, Difco™ Mueller–Hinton BD®, BBL™ Blood Agar Base BD, and Chromagar™ Orientation (France). Plates were incubated at 37 °C for 24–36 h. Bacterial identification was carried out by Gram staining, biochemical tests, and Chromagar evaluation. Antibiograms were performed according to bacterial species identified, and after 24 h, antibiotics were selected based on sensitivity (inhibition zone diameter).

Intrauterine infusion of N-acetylcysteine (NAC)

Cows in the treatment group (T-NAC = 5) received an intrauterine infusion of 40 mL of 2% NAC (Fluimucil® 300 mg/3 mL, Zambon – Italy) administered with a plastic pipette at 6:00 a.m. Twelve hours later, the appropriate intrauterine antibiotic, based on antibiogram results in

positive cases, was administered. The procedure was repeated for three consecutive days.

Intrauterine infusion of antibiotics

Antibiotics for intrauterine administration were selected according to antibiogram sensitivity profiles. Twelve hours after NAC administration (6:00 p.m.), intrauterine antibiotic treatment was applied. This procedure was performed for three consecutive days.

Statistical analysis

Data were organized in an-Excel spreadsheet and analyzed using InfoStat 2020 software. Descriptive measures such as mean, median, and standard deviation were calculated to summarize and characterize data distribution. To evaluate differences between two related conditions, a paired Student's t-test with two-tailed contrast was applied, allowing statistical inference on the presence of significant differences between conditions.

The results of the reduction in %PMN-N in cows with SE treated with NAC are presented in Table 2.

Table 2. Comparison of %PMNs before and after treatment with NAC

GROUP	ID	Bacteria identified	%PMNs	
			Before	After
T-NAC	1		8	2
	2	- <i>Staphylococcus epidermidis</i>	12	3
	3	- <i>Escherichia coli</i>	17	5
	4		9	1
	5		11	4
GROUP	ID	Bacteria identified	%PMNs	
			Before	After
nT-NAC	8	- <i>Staphylococcus aureus</i>	16	18
	9	- <i>Staphylococcus epidermidis</i>	12	21
	10		8	13
	13		13	14
	14		10	8

In Table 2, paired samples are compared before and after treatment with or without NAC.

The %PMN-N values in the T-NAC group decreased, and this reduction was statistically significant ($p < 0.05$).

In contrast, the nT-NAC group showed no decrease, and results were not statistically significant ($p > 0.05$). In Figure 3, the effect of NAC before and after treatment is illustrated. In the T-NAC group, %PMN

values decreased below the diagnostic threshold for PMN-N following treatment, whereas the nT-NAC group did not show relevant changes.

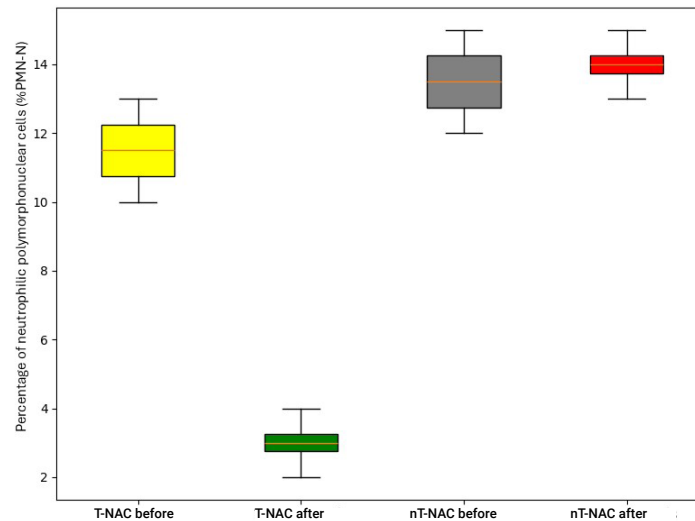


Figure 3. Comparison of the T-NAC and nT-NAC groups. Distribution of the percentage of polymorphonuclear neutrophils (PMNs) in cows with subclinical endometritis before and after treatment. The boxplots represent treated (T-NAC) and untreated (nT-NAC) groups, before (gray and yellow) and after (green and red) NAC infusion. A significant reduction in %PMN was observed in the post-treatment T-NAC group (green), while the nT-NAC group showed no relevant changes.

DISCUSSION

The %PMN-N values in samples obtained before and after NAC treatment were statistically different ($p < 0.05$), whereas in cows without NAC treatment, differences were not statistically significant ($p > 0.05$). A review of the literature reveals that studies on the use of NAC to reduce PMN-N are scarce; however, our results are consistent with the findings of Tras *et al.*²³, who suggested that N-acetylcysteine (NAC) may be an effective therapeutic option in the treatment of reproductive tract infections accompanied by purulent discharge, such as clinical cases of endometritis. This possibility is supported by the pharmacological properties of NAC, including its good bioavailability, antioxidant and mucolytic capacity, and low cost, making it an accessible and potentially useful alternative in both clinical and veterinary contexts.

In humans, Anastasi *et al.*¹ reported that oral NAC administration reduced pain associated with endometriosis and the size of endometriomas. Furthermore, NAC decreased serum levels of CA-125 (a biomarker of endometriosis and pelvic inflammatory disease in humans)²² and improved fertility outcomes in patients with endometriosis.

NAC is a thiol compound, a precursor of L-cysteine and reduced glutathione, and a known mucolytic agent. Its antioxidant properties arise from its ability to scavenge free radicals by interacting with reactive oxygen species (ROS) such as OH and H₂O₂. In addition, NAC serves as a source of sulfhydryl groups within cells, further supporting its role as a reducing and antioxidant agent.

The beneficial effects of NAC on fertility may be explained by its antiproliferative and

antioxidant effects on tissues, facilitating the shift from proliferation to differentiation, and by its downregulation of inflammatory genes and proteins¹³. NAC, as a glutathione precursor, has well-established antioxidant and anti-inflammatory properties mediated by both direct and indirect mechanisms. The direct effect is related to the free thiol group that scavenges ROS, while the indirect effect involves its ability to enter cells, react with glutamic acid and glycine, and increase intracellular glutathione levels, which in turn reduce ROS¹¹.

Previous studies in both animal and human tissues have demonstrated that NAC significantly reduces the size of endometriotic lesions and alleviates pain symptoms¹⁴. NAC has a relatively high pKa (9.51 at ionic strength I = 1 M; 9.87 at I = 0.02 M) compared with other thiols, which means its reactivity toward most electrophiles and oxidants is lower. Nonetheless, NAC reacts rapidly with specific radicals such as OH, NO₂, and CO₃, explaining its antioxidant action.

NAC provides protection to various cellular systems. Cysteine transport is mainly mediated by Na⁺-dependent neutral amino acid systems. However, NAC does not require active transport, as it is a membrane-permeable cysteine precursor. Esterification of the carboxyl group of NAC to produce N-acetylcysteine ethyl ester further increases its lipophilicity. Once free NAC enters the cell, it is hydrolyzed to release cysteine, a key precursor of glutathione (GSH). GSH synthesis is limited by substrate availability, with cysteine often being the rate-limiting precursor. GSH plays an essential role in protecting against oxidative damage caused by ROS. Glutathione reductase maintains GSH in its reduced form, becoming activated under oxidative stress. The destruction of H₂O₂ and hydroperoxides is catalyzed by GSH peroxidase⁷.

Finally, NAC treatment may contribute to ROS elimination. Pengjie *et al.*¹² demonstrated that NAC significantly reduced the expression of mitochondria-dependent apoptotic proteins, restored mitochondrial membrane potential, and normalized energy synthesis in bovine endometrial epithelial cells. Taken together with the present findings, which demonstrate reduced %PMN-N, these considerations support NAC as a potential local therapeutic (via intrauterine infusion) in the treatment of SE, possibly due to its antioxidant action.

CONCLUSION

Intrauterine administration of N-acetylcysteine (NAC) in cows with subclinical endometritis (SE) resulted in a significant reduction in the percentage of polymorphonuclear neutrophils (%PMN-N) in the treated group (T-NAC), reaching values below the diagnostic threshold ($\geq 5\%$), whereas no relevant changes were observed in the untreated group (nT-NAC). This experimental finding was validated through paired-sample statistical analysis, showing a significant difference ($p < 0.05$) in the treated group.

The mechanism of action of NAC is based on its mucolytic and antioxidant capacity. As a glutathione precursor, NAC reduces oxidative stress in the endometrium, decreases the expression of mitochondria-dependent apoptotic proteins, and restores mitochondrial membrane potential. Moreover, NAC breaks disulfide bonds in mucoproteins, facilitating secretion clearance and improving the uterine environment for tissue recovery.

From a clinical perspective, NAC can be incorporated as a complementary treatment in bovine reproductive protocols, particularly in cows with SE diagnosed by endometrial cytology. Its administration prior to antibiotic therapy improves treatment efficacy, reduces inflammation, and increases pregnancy rates without generating residues in edible tissues. These findings support the use of NAC as a safe and effective tool in bovine reproductive medicine.

CODE OF ETHICS

The authors declare that the presented study was carried out in accordance with the Code of Ethics for animal experiments, as reflected in the regulations: https://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm

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