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ASCORBIC ACID IN COMBINATION WITH AMOXICILLIN IMPROVES ACUTE ENDOMETRITIS IN MURINE MODELS

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ABSTRACT

Endometritis, refers to uterine lining inflammation which is a common postpartum infection that can cause uterine rupture in pregnancy. This study investigated the outcome of combining ascorbic acid (AA) with amoxicillin (AMX) in a mouse model of endometritis. Regular cycling mice were inoculated with *E. coli* and placed in different experimental groups. After *E. coli* inoculation, treatment was administered for five days. Group 1 received AA (100 mg/kg) and AMX (500 mg/kg); group 2 received AA (1000 mg/kg) and AMX (500 mg/kg) and group 3 received ibuprofen (400 mg/kg) and AMX (500 mg/kg). Estrous cycling, body temperature, and body weights were assessed. At the end of the experiment, blood samples and uteri were collected and assessed. Uterine contractility was also measured as an indicator of function. AA (1000 mg) + AMX (500 mg/kg) combination restored estrous cyclicity, improved body temperature, restored uterine organ weights, and increased leukocytic parameters. The uterine architecture was also restored, and uterine function was improved. This study shows that combination therapy with AA and AMX provides a better therapeutic option in the management of endometritis.

KEYWORDS: **Endometritis, Ascorbic Acid, Combination Therapy, Amoxicillin, Uterus, Ibuprofen.**

INTRODUCTION

Endometritis is a pelvic inflammatory disease for which current antibiotic therapy produces variable outcomes [1]. Endometritis is characterized by inflammation of the endometrium and can lead to poor implantation and, consequently, infertility [2]. Endometritis occurs from the upward travel of bacteria from the cervix and vagina. Predisposing factors include caesarean section, prolonged labor, uterine membrane rupture, and obesity [3]. The route of delivery is, however, considered the most significant risk factor [2]. Complications also frequently arise from endometritis, which also leads to reduced fertility. These complications include sepsis, abscesses, as well as uterine necrosis [4]. Current treatment involves the use of antibiotics, such as amoxicillin, doxycycline, or gentamicin [5, 6]. However, treatment with antibiotics has produced variable outcomes [1]. The variable outcome of antibiotic therapy for endometritis has, therefore, necessitated an investigation into more efficient therapies or treatment regimen. Reactive oxygen species (ROS) are produced during endometritis and occur due to the action of pro-inflammatory mediators, which are dependent on the content of ROS [7]. Therefore, targeting the

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bacteria or causative organism alone may not result in complete resolution of endometritis and may have resulted in the variable outcomes noticed.

In our earlier study, we reported that single therapy administration of ascorbic acid (AA) at doses of 100 and 1000 mg/kg improved acute endometritis in mouse models and restored uterine function [8]. We now hypothesize that the combination of AA and AMX rather than single therapies of each will improve endometritis further, a practice that may be easier to implement in clinical settings.

Combination therapy, which involves the use of more than one drug for the treatment of a single disease condition, is becoming mainstream therapy since some diseases result from diverse range of mechanisms such that targeting a single component may no longer be sufficient [9].The utilization of antioxidants, in combination with antibiotics, is currently being proposed [10] because oxidative stress occurs at several diseases onset [11]. Therefore, new therapies of inflammatory conditions by targeting oxidative pathways are suggested [10].

The current study was aimed at evaluating the benefits of combining AMX and AA in the therapy of acute endometritis in mouse models.

MATERIALS AND METHODS

Materials

Amoxicillin (Glaxosmithkline Beecham, UK), ascorbic acid (Nomagbon pharmaceuticals, Nigeria); methanol and formal saline (Pharmatrends, Benin City, Nigeria), ibuprofen (Sun Pharmaceuticals, Nigeria), nutrient broth (Lab M batch No. 113693/308), *Escherichia coli* (clinical isolate from University of Benin Teaching Hospital, Edo State. Nigeria).

Animals

Female albino mice, (25.0-32.0 g), ten weeks of age, were maintained at the animal unit of the Department of Pharmacology and Toxicology, University of Benin, Nigeria. They were housed in cages at an environmentally controlled room temperature of approximately 30 ± 4°C and natural lighting conditions. Relative humidity ranged from 75-80% within the period. The animals were acclimatized to these conditions. Handling was done according to standards of the Public Health Service policy on humane care and use of Laboratory Animals [12, 13]. Ethical permissions were also obtained from the Faculty of Pharmacy Ethical Committee, University of Benin, Nigeria. The animals were adequately cared for and fed a standard diet of animal pellets and clean tap water.

Organism

A clinical isolate of *Escherichia coli* (*E. coli*) was used in this study. The isolate had been shown to produce persistent endometritis [14] in mice [8].

Mouse uterine endometritis model

The method by Bafor et al. [8] was used. Briefly, E. *coli* was inoculated into a nutrient broth (10 mL) and incubated for 24 h at 37°C. The broth culture was diluted serially, and 50 µL of each dilution was plated in Petri dishes. After the 24 h incubation period, the pure culture nutrient broth of *E. coli* was prepared using sterile normal saline to give an inoculum of *E. coli* 1.0 x 10^5 (CFU/mL). In subsequent experiments, mice with regular estrus cycles were placed in three experimental groups: group 1 received AA (100 mg/kg) and AMX (500 mg/kg), group 2 received AA (1000 mg/kg) and AMX (500 mg/kg), and group 3 received IBP (400 mg/kg) and AMX (500 mg/kg). Drug administration was instituted two days post-inoculation of *E. coli* (50 µL/mice *E. coli* intravaginally, 10^5 CFU/mL). All drugs were administered orally via a feeding syringe for five days, once daily. AMX was administered first, and 30 min afterward, AA or IBP was administered. This was done to avoid any possible interaction between the drugs in the gastrointestinal system. Drug administrations were done in the morning between 9-10 am daily.

Vaginal lavage collection

Animals that showed regular 5-6 days estrus cycles were utilized. Vaginal smears were collected before inoculation, and daily throughout the study with the aid of a sterile micropipette contained normal saline (0.1 mL) [15]. The collected smears were dried, fixed in cold methanol and stained with a drop of gentian violet and examined under the microscope.

Body temperature and weight measurement

Body temperatures were assessed rectally on day 0 (before inoculation day) and after 24 h after each daily drug administration [16]. This was done using a digital probe thermometer (model Panlab-0331, China). The body weights were taken on day 0 and day 6 (post-drug administration). On day 6, the animals were anesthetized by diethyl ether inhalation. Blood and tissue samples were collected, as described below.

Blood sample and organ collection and analysis

Blood samples collected via cardiac puncture (approximately 0.5 mL) were placed in Na^+ EDTA (1.5 mg) tubes for hematological analyses [17]. Hematological assays were performed using an automated hematology analyzer (SYSMEX KX-21N Japan). Immediately after collection of blood samples, the animals were exsanguinated, and the abdomen opened longitudinally. The uterine horns were isolated, carefully removed, and weighed. The uterus was split to give two horns, and one horn was placed in an aerated warm physiological salt solution for *ex-vivo* contractility assays. The other horn was fixed in 10% neutral buffered formal saline (1:10 v/v) [14] and submitted for histopathological analysis.

Ex-vivo uterine function assay

The uterine horn was divided into two segments of about 0.5 cm lengths. The segments were mounted longitudinally in a 10 mL organ bath containing physiological salt solution (PSS) of the following composition in g/5L distilled water: NaCl 45.0; NaHCO3 2.5; D-glucose 2.5; KCl 2.1; $CaCl_2.2H_2O$ 1.32 and maintained at a constant temperature of 37°C, air was supplied with an aerator (RS-180, Zhongshan, China). The lower end of the uterine tissue was attached to a tissue holder while the upper end was connected to an isometric force-displacement transducer (Model 7003-E) connected to a Data capsule digital recorder system (Model 4050 Ugo Basile, Italy). The set up was equilibrated under resting tension of 5 mN for 15 min or till regular contractions were obtained before the commencement of the experimental protocol. The differential force and frequency of spontaneous contractions in the longitudinal muscle layer of the uterine tissues were recorded after equilibration for 30 min. Contractions were analyzed by averaging the force and frequency of contractions during the last 10 min [8].

Statistical analyses

Data are presented as mean ± standard error of the mean (SEM). One-way analysis of variance was conducted where appropriate and followed by Tukey's multiple range tests, and the Student's two-tailed t-test performed where appropriate also. Statistical tests were performed with the aid of GraphPad Prism version 5.00 for La Jolla California USA, $P < 0.05$ was considered significant in all cases [14].

RESULTS

Estrous cycle analysis

A disruption to the normal estrous cycle was observed under endometritis conditions in the different groups' post-inoculation. Administration of AA 100 mg/kg + AMX 500 mg/kg showed some resolution, evidenced by the presence of few cornified cells with little or no leukocytic cells seen at the end of the study (Figure 1a-c). Regular estrus cycling was better restored in the presence of AA 1000 mg/kg + AMX 500 mg/kg, evidenced by the dominant presence of nucleated epithelial cells and few cornified cells at the end of the study (Figure 1d-f). The IBP 400 mg/kg + AMX 500 mg/kg showed dominant leukocytic infiltration at the end of the study (Figure 1g-i).

Figures 1a, b and c represent AA 100 mg/kg and AMX 500 mg/kg groups. (a) Before treatment while in endometritic state. Cells were mainly cornified (block arrow); (b) Day 3 of treatment. Nucleated epithelial cells (slim arrow) and red blood cells observed (open arrow). (c) Cornified cells (block arrow) dominant on Day 6.

Figures 1d, e and f represent AA 1000 mg/kg and AMX 500 mg/kg groups. (d) Before treatment while in endometritic state. Cells were mainly cornified (block arrow), with some leukocyte infiltration (block arrowhead) and few nucleated epithelial cells (slim arrow); (e) Day 3 of treatment with dominant nucleated epithelial cells (slim arrow) and few cornified cells (block arrow); (f) Combination of cornified cells (block arrow) and nucleated epithelial cells (slim arrow) observed.

Figures 1g, h and I represent IBP 400 mg/kg and AMX 500 mg/kg groups. (g) Before treatment while in endometritic state with dominant leukocytic infiltration (block arrowhead) and few nucleated epithelial cells (slim arrow); (h) Day 3 of treatment with cornified cells (block arrow) and few nucleated epithelial cells (slim arrow); (i) Day 6 with dominant nucleated epithelial cells (slim arrow) and few red blood cells (open arrow).

Body temperature analysis

The body temperature was increased at the end of the study in all the groups (Table 1). There was no significant difference ($p = 0.144$) in body temperatures in the IBP+AMX group at the end of the study, and a significant increase ($p < 0.05$ and $P < 0.001$) in body temperatures in the groups treated with AA100 + AMX and AA1000+ AMX respectively (Table 1). AA 1000 mg/kg + AMX 500 mg/kg combination produced the most significant increase in body temperature.

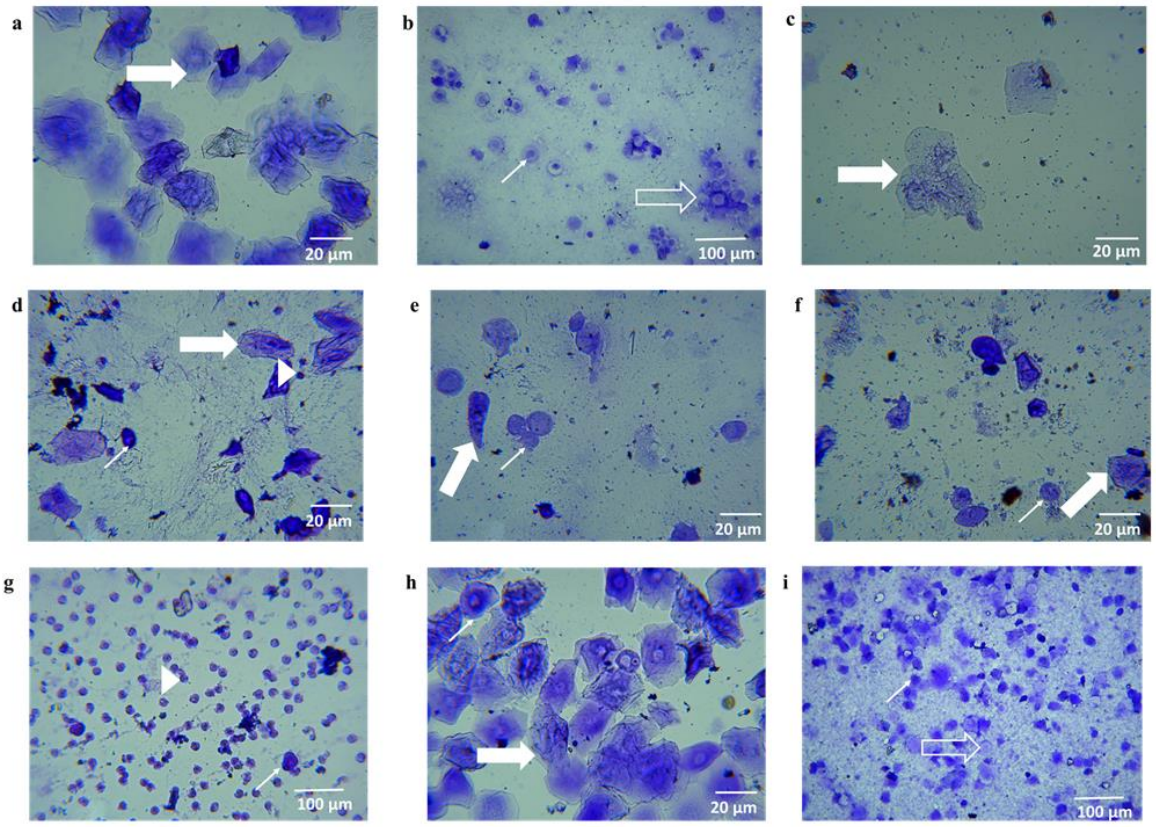

Figure 1: Representative photomicrograph of vaginal smears x100 and x 400.

Table 1: Temperature ranges in *E. coli*-induced endometritis mouse models treated with combination of ascorbic acid and amoxicillin; amoxicillin and ibuprofen.

Days	Groups (°C)		
	AA 100 + AMX	AA 1000 + AMX	IBP + AMX
1	36.65 ± 0.56	36.73 ± 0.30	37.60 ± 0.24
2	37.40 ± 0.75	37.75 ± 0.17	37.63 ± 0.43
3	37.20 ± 0.42	37.60 ± 0.35	36.93 ± 0.37
4	37.58 ± 0.39	38.30 ± 0.32	37.15 ± 0.67
5	38.60 ± 0.35	38.58 ± 0.21	37.90 ± 0.21
6	38.50 ± 0.31*	39.30 ± 0.08***	38.47 ± 0.33

n = 4 animals, *p<0.05; ***p<0.001 compared to day 1 of same group; AA = ascorbic acid; AMX = amoxicillin; IBP = Ibuprofen.

Body and uterine organ weight analysis

There were no statistically significant changes ($p = 0.8477$) in the body weights of the animals during and at the end of the study (Figure 2a). However, a slight increase in body weight occurred in the AA 100 mg/kg + AMX 500 mg/kg group (Figure 2a).

The AA 1000 mg/kg + AMX 500 mg/kg group showed significantly lower ($p < 0.05$) uterine weights compared to the other groups in this study (Figure 2b). The uterine weights for the AA 100 mg/kg + AMX 500 mg/kg were slightly lower than those of the IBP + AMX group (Figure 2b).

Histological evaluation of the endometrium

Stromal hyperplasia was clearly evident in the AA 100 mg/kg + AMX 500 mg/kg group as well as the IBP 400 mg/kg + AMX 500 mg/kg (Figure3a and c respectively). No sign of hyperplasia was evident in the AA 1000 mg/kg + AMX 500 mg/kg group (Figure3b). There was also no leukocytic infiltration in the AA 1000 mg/kg + AMX 500 mg/kg group, which were evident in the other groups (Figure 3c).

Ex-vivo uterine function analysis

Uterine tissues from the AA 100 mg/kg + AMX 500 mg/kg and the IBP 400 mg/kg + AMX 500 mg/kg groups had more uterine activity than the AA 1000 mg/kg + AMX 500 mg/kg group (Figure 4a-c). This was also observed on analysis of the amplitude and frequency of contractions where it was shown that the force of contractions where increased in AA 100 mg/kg + AMX 500 mg/kg and IBP 400 mg/kg + AMX 500 mg/kg groups compared to the AA 1000 mg/kg + AMX 500 mg/kg group (Figure 4d and e).

Hematological analysis

A significant decrease ($p < 0.001$) in AA1000 + AMX group and a significant increase ($p < 0.001$) in IBP+ AMX group occurred only in the platelet (PLT) (Table 2). White blood cells (WBCs) increased in the AA 1000 mg/kg + AMX 500 mg/kg compared to the other groups (Table 2). An increase in the lymphocyte (LYM) and granulocyte (GR) was evident in the AA 1000 mg/kg + AMX 500 mg/kg. There was elevated red blood cell (RBC) and hemoglobin (Hgb) levels.

DISCUSSION

Infections disrupt estrous cycling [18] and induce a state of persistent estrus characterized by the presence of cornified cells [18] which can result in infertility or low fertility rates [19]. A state of high leukocytic infiltration also indicates the presence of infection. The interactions between the endocrine and the immune do occur such that the immune system can alter the endocrine system function which can be detected in vaginal smears [20]. This is important since vaginal smears can be used to indicate the state of the reproductive cycle [21].

As reported earlier, most rodents display decreases in their body temperature in the presence of persistent infection [8, 22], a mechanism that serves to attenuate the inflammatory response to the infection while improving survival response [23]. The reduction in temperature also occurs in tandem with decreased leukocytes in mice as an indication of sepsis [24], and is responsible for the low temperatures at the start of this study when endometritis was instituted. Increased temperatures which occurred at the end of the study, therefore supports an improvement of the endometritis condition and additionally supports the combination of AA and AMX for better attenuation of endometritis. The increased temperature in the current study is an improvement over our earlier study, where single therapies of either AA or AMX were used and no significant differences in temperatures were observed [8].

Bacterial infection causes uterine cell proliferation and consequently hyperplasia [25] as well as increased uterine weight [8]. Therefore, the lower weights experienced in the AA and AMX combination are indicative of uterine cell proliferation inhibition. Our earlier study showed that AMX caused hyperplasia of the uterine cells in endometritis [8] and may account for the larger weights observed in the other groups while also supporting the effectiveness of AA 1000 in overcoming the AMX-induced hyperplasia under these conditions. The high uterine weights in AA and AMX combination may also have contributed to the increase in body weights observed and is also indicative of continuing infection [26]. This observation suggests that the infection was not completely cleared in the affected groups while complete infection occurred in the AA 1000 mg/kg + AMX 500 mg/kg group where there was no evidence of hyperplasia. The effectiveness of the combination is also supported by the absence of leukocytic infiltration.

Our earlier study had shown that endometritis increased the frequency and tension of uterine contractility [8], which may have occurred in response to bacterial materials that go on to potentiate calcium entry and consequently muscle cell contraction [27]. The slightly increased contractions may therefore indicate an incompletely cleared infection, which was better resolved by the AA 1000 mg/kg + AMX 500 mg/kg treatment. The

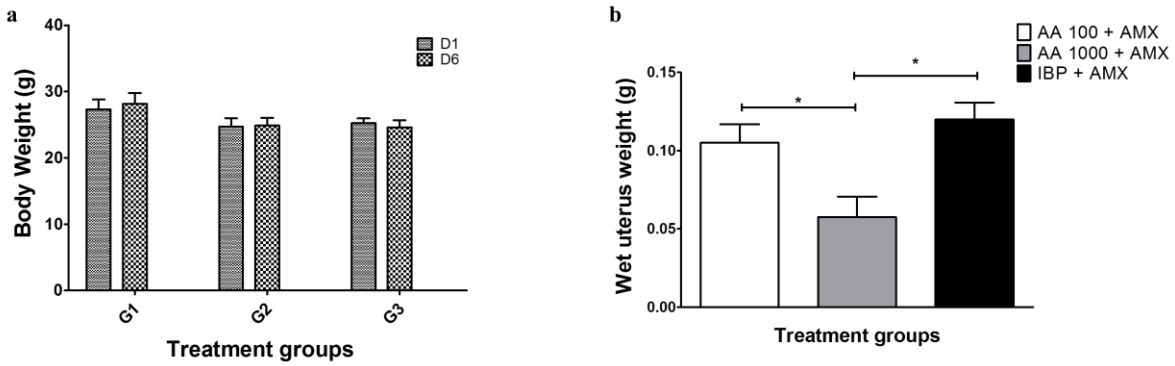

Figure 2: Body and uterine weights of mice. There were no significant changes in the body weights before and after drug administration. G1 = group 1 (AA 100 mg/kg + AMX 500 mg/kg), G2 = group 2 (AA 1000 mg/kg + AMX 500 mg/kg), G3 = group 3 (IBP + AMX 500 mg/kg) (a). Wet uterine weights from E. coli-induced endometritis murine models treated with ascorbic acid, amoxicillin, and ibuprofen (b). No statistically significant differences were observed in the uterine weights. However, the group treated with AA 1000 mg/kg + AMX 500 mg/kg showed the lowest uterine weights, and this was followed closely by the IBP + AMX group. The AA 100 mg/kg + AMX 500 mg/kg groups had the largest uterus weights. n= 4 animals in all groups.

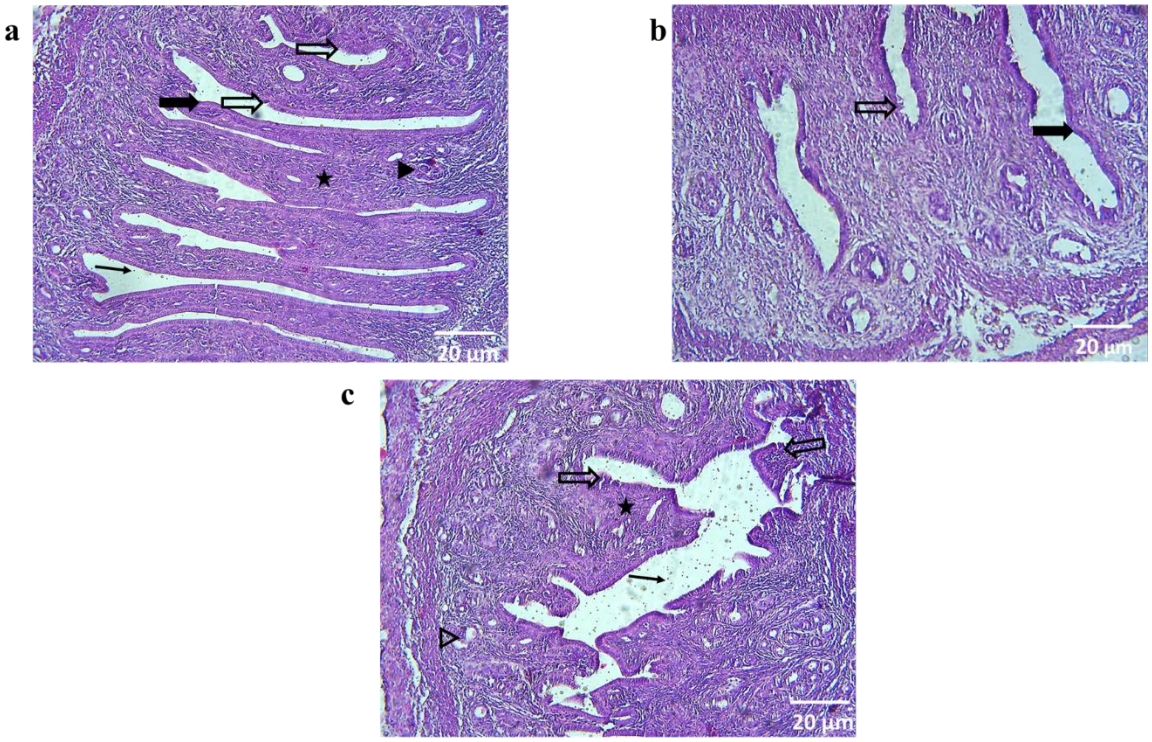

Figure 3: Representative images of the histopathological assessment of changes in the uterus. H&E x 40 of mice treated with AA 1000 mg/kg and AMX 500 mg/kg. The endometrial lining was largely intact (block arrow) with few mild sloughing observed (open arrow).

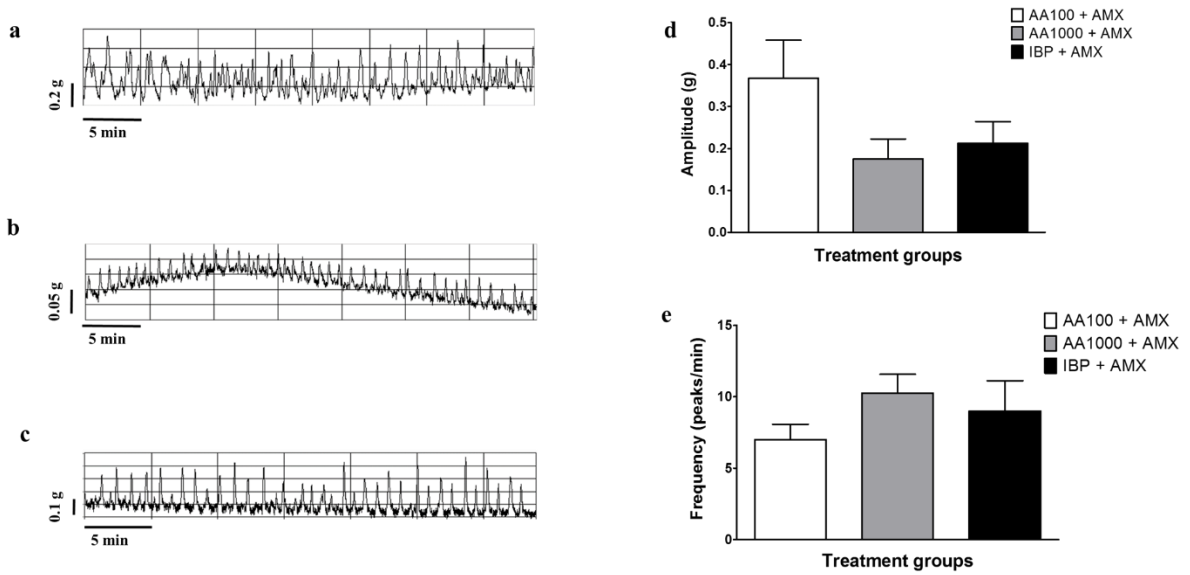

Figure 4: Original representative recordings showing intrinsic contractions of the uterus from mice with *E. coli*-induced endometritis on combination treatments. Panel a represents the group that received AA100 mg/kg and AMX 500 mg/kg; panel b represents the group that received AA 1000 mg/kg and AMX 500 mg/kg, and panel c represents the group that received IBP 400 mg/kg and AM 500 mg/kg. Analysis of the amplitude and frequency of intrinsic uterine contractions from mice with *E. coli*-induced endometritis on combination treatments (e-f). No statistically significant differences were observed in the amplitude of contractions among the groups treated (e). No statistically significant differences were observed in the frequency of contractions among the groups treated (e). n = 4 animals.

Table 2: Haematological indices in the combination groups.

Haematological parameters	**Groups**		
	AA100 +AMX	**AA1000 + AMX**	**IBP +AMX**
WBC[a]	5.43 ± 2.28	7.10 ±1.67	4.90 ± 1.46
RBC[b]	7.99 ± 1.01	8.58 ± 0.75	10.57 ± 1.19
HGB[c]	13.13 ± 0.74	14.13 ± 1.00**^**	15.00 ± 0.43
HCT[d]	27.13 ± 7.41	30.88 ± 7.63	34.85 ± 6.39
MCV[e]	47.20 ± 2.49	44.73 ± 0.33	43.23 ± 0.34
MCH[f]	16.80 ± 1.07	16.50 ± 0.55	16.40 ± 0.66
MCHC[g]	35.63 ± 0.99	36.93 ± 1.02	36.93 ± 1.62
PLT[h]	553.30 ± 27.13	162.31 ± 29.60***	216.30 ± 38.59***
LYM (x 10^{-3})[i]	4.27 ± 1.42	5.68 ± 1.47	2.95 ± 0.91
MO[j]	0.63 ± 0.48	0.63 ± 0.28	0.63 ± 0.34
GR[k]	0.57 ± 0.42	0.80 ± 0.23	0.50 ± 0.07
RDW[l]	15.47 ± 1.46	17.48 ± 0.56	20.35 ± 2.22
PCT[m]	0.38 ± 0.06	0.13 ± 0.06*	0.18 ± 0.05
MPV[n]	7.43 ± 0.84	6.13 ± 0.22	6.45 ± 0.39
PDW[o]	8.37 ± 0.67	7.38 ± 1.38	7.84 ± 0.44

a = white blood cell (x 10^3 mm^{-3}); **b** = red blood cell (x 10^6 mm^{-3}); **c** = haemoglobin concentration (g/dl); **d** = haematocrit (%); **e** = mean corpuscular volume (fl); **f** = mean corpuscular haemoglobin (pg); **g** = mean corpuscular haemoglobin concentration (g/dl); **h** = platelet (x 10^3 mm^{-3}); **i**= lymphocytes (x 10^3 mm^{-3}); **j** = monocyte (x 10^3 mm^{-3}); **k** = granulocyte (x 10^3 mm^{-3}); **l** = RDW = red cell distribution width (fl); **m** = platelet count (x 10^3 mm^{-3}); **n** = mean platelet volume (x 10^3 mm^{-3}); **o** = platelet distribution width (x 10^3 mm^{-3})

^p<0.05 increase compared to AA100 + AMX; *p<0.05; *** = p<0.001 decrease compared to AA100+AMX. n= 4 animals.

contractions were also regular with reduced force compared to the previous study which involved single therapies [8].

PLT function is altered in disease and inflammatory conditions [28]. PLTs are known to accumulate AA which is vital to PLT function [29]. However, an inverse relationship between plasminogen activator and AA exists and may account for the decreased levels of PLT in the AA and AMX combination, which is considered beneficial to the body system overall as it reduces the risk of cardiovascular events [30]. WBCs have been shown to decrease considerably in endometritis mouse models [8] and only improved on treatment. The decrease in WBCs with AA1000 and AMX combination provides better therapy for endometritis compared to the other groups and also compared to single therapies [8]. Earlier studies had also shown an improvement in the lymphocyte (LYM) and granulocyte (GR) hematological parameters on treatment [8], which was also observed in this study but mostly increased in the AA 1000 mg/kg + AMX 500 mg/kg. The improved lymphocytes and granulocytes levels were attributed to the accumulation of AA in WBCs, which provides added cell protection [31] and increased clearance of the infecting organism. Therefore, the use of AA 1000 mg/kg + AMX 500 mg/kg results in optimal endometritis therapy. Increased red blood cell (RBC) and hemoglobin (Hgb) levels were observed in this study, as in our earlier study again promoting the beneficial effect of the combination therapy.

CONCLUSION

In summary, this study has shown that the use of a combination therapy utilizing oral administration of AA 1000 mg/kg and AMX 500 mg/kg is more beneficial in ameliorating endometritis and improving uterine function compared to single therapies of either drug. This study also shows that the combination of AA and an antibacterial is more efficient than combining anti-inflammatory and antibacterial in the therapy of endometritis. Clinical trials on this combination is therefore suggested for further confirmation.

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