

Full Length Research Paper

The use of metronidazole and activated charcoal in the treatment of diarrhea caused by *Escherichia coli* 0157:H7 in an *in vitro* pharmacodynamic model

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Accepted 1 September, 2011

Activated charcoal (AC) has been utilized in the absorption of toxins from the intestinal tract of animals as well as humans. Some studies however have implied microbial adsorption at the interstices of AC surface. Activated charcoal has also been suggested and used as a treatment option for diarrhea. Metronidazole, a nitroimidazole derivative has been successfully utilized in the treatment of Clostridium difficile-associated diarrhea (CDAD) as well as anaerobic infections. It has little or no activity against *Escherichia coli* 0157:H7. The purpose of this study is to compare the activity of metronidazole-AC combination to that of AC alone in the management of *E. coli* 0157: H7. Utilizing an *in vitro* pharmacodynamic model in triplicate, *E. coli* 0157:H7 was exposed to peak concentrate of 32 mg/L of metronidazole daily. Time kill curve was analyzed for time to 3 log killing slope and re-growth of the micro-organism. Minimal inhibitory concentrations (MICs) for pre-exposure to metronidazole were 1 mg /L. Metronidazole alone did not decrease the starting inoculums of *E. coli* 0157:H7. However, the metronidazole - AC combination achieved a 2 – log killing against *E. coli* 0157:H7 as well as a significant reduction in the starting inoculums below the lower limit of bacterial counting accuracy (LLA) when AC alone was used. The metronidazole -AC MIC for *E.coli* increased 5 fold when compared to utilization of AC alone or metronidazole alone. Metronidazole -AC will be useful in the treatment of mild to moderate diarrhea associated with *E.coli* 0157:H7.

Key words: Metronidazole, activated charcoal, *in-vitro* pharmacodynamic model, *Escherichia coli* 0157:H7.

INTRODUCTION

The predominant micro-organisms in most stool isolates as well as the gastrointestinal tract (GIT) in developing and underdeveloped countries including the aerobic, enterobacteria *Escherichia coli* and varying anaerobic organisms (CDC Division of Bacterial and Mycotic Diseases, 2010). The strains associated with diarrhea disease include enterotoxigenic, enteropathogenic, enteroinvasive and enteroheamorrhagic *E. coli* (EHEC). The EHEC strain causes diarrhea and is mainly associated with the serogroup 0157:H7 (Todar, 2002). Infection occurs via ingestion of contaminated food and

water. Diarrhea infections may also be characterized by a mixture of aerobic and anaerobic bacteria where synergistic activity facilitates the infectious process by promoting the proliferation of anaerobic bacteria (Stein, 1998).

Metronidazole is commonly utilized for the treatment of diarrhea owing to its activity against anaerobic organisms (American Hospital Formulary Service, 2000). Activated charcoal has been utilized in treatment of poisoning, as well as an absorbent of toxins produced in the intestine and has been suggested to be useful in treatment of diarrhea associated with *E. coli* via adsorption of the organism (Naka et al., 2001; Haley et al., 2006)

This study compares the activity of the combination of metronidazole - AC given daily; activated charcoal alone and metronidazole alone in an *in vitro* pharmacodynamic

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model containing *E. coli* as the test organism.

MATERIAL AND METHOD

Six concentration time kill curve was used as a representation of the adsorptive effect of AC on the organism. The *E. coli* 0157:H7 was exposed to 400 mg metronidazole, metronidazole-AC combination or AC every 24 h. A growth control experiment was conducted in triplicate for *E. coli* ATCC 25922.

In vitro model

The experiment was conducted using a modification of the *in vitro* model previously described (Zabinski et al., 1993; Hermsen et al., 2005) placed in a Bactron IV anaerobic chamber (Sheldon Manufacturing, Cornelius, Oregon). This system supports an anaerobic environment which operates with an anaerobic chamber mixed gas of 5% hydrogen, 10% carbon dioxide, and 85% nitrogen (Zabinski et al., 1993; Hermsen et al., 2005), however, these gases were not employed as an aerobic environment was required for *E. coli* growth. An inoculum of the organism was introduced into each chemostat followed by a bolus of metronidazole alone; metronidazole -AC and AC alone. Utilizing the desired concentration, each experiment and control was run in triplicate for 24 h.

Bacteria

Clinical isolates of *E. coli* 0157:H7 obtained from human diarrheal stool isolate as well as *E. coli* ATCC 25922 strain was used. Several colonies of each isolate was incubated aerobically overnight in 50 ml of broth. The overnight culture was diluted down to obtain a one in ten dilution in fresh warm broth approximately half an hour before the experiment, in order to allow the organism to attain exponential growth. An approximate amount of the culture compared to a 0.5 McFarland equivalent turbidity standard was added to each chemostat, giving the resultant initial bacterial inoculum to be 10^6 CFU/ml for all the experiment.

Susceptibility testing

Susceptibility testing was performed in triplicate for the clinical isolate and the control isolate (*E. coli* 0157:H7 and *E. coli* ATCC 25922, respectively) prior to the concentration time kill experiments at 24 h post exposure. Broth micro dilution method in cation adjusted Mueller – Hinton broth was carried out with an inoculum of 10^5 CFU/ml. 24-well trays inoculated with *E. coli* were incubated for 20 h at 36°C in an oven.

Colonies present at 24 h were counted and stored at 3°C until required for subculture on fresh agar plates.

Antibiotics

Stock solution of metronidazole BP (Sigma Aldrich St. Louis Mo) was prepared and kept refrigerated at 3°C. A dose of 400 mg metronidazole was administered in a 24- h period.

AC was purchased from BDH chemical Ltd Poole England Batch No 5607050A. It was sterilized by putting it in an oven at 100°C for 1 h and then left to cool in a sterile dessicator and 200 mg dose was administered every 24 h either alone or in combination with 400 mg metronidazole. High performance liquid chromatography, HPLC (Scientific Research Consortium, Incorporated Minnesota)

methodology used for the assay of metronidazole was the method reported by Jessa et al. (1996). The curve was linear over a range of 0.2 to 26 mg/L ($r^2 \geq 0.994$). The interday coefficient of variance was 8.2%.

Test tube analysis

This was performed utilizing the *E. coli* 0157:H7 and *E. coli* ATCC 25922 strains in triplicate. From tubes labeled one to ten as reflected in Table 1 increasing concentrations of both AC alone and metronidazole – AC were administered to determine the effect of concentration on the starting inoculum of *E. coli*. After 24 h, 1 ml samples were removed from the test tube model for quantification of the bacterial density by serial dilution techniques. Bacterial counts were determined by 1:10 serial dilution of the sample and plated on trypticase soy agar (TSA) for *E. coli*. The residual AC was washed out to recover micro organism if any and the residue plated again on TSA.

Pharmacodynamics

At eight predetermined time points, 0, 1, 2, 3, 4, 6, 12 and 24 h, 1 ml sample were removed from the model for quantification of the bacterial density by serial dilution techniques. Bacterial counts were determined by 1:10 serial dilution of the sample and plated on trypticase soy agar (TSA) for *E. coli*. After aerobic incubation for 24 h, at 37°C, the numbers of *E. coli* CFU on each plate were visually counted lower limit of bacterial counting accuracy (LLA) was taken as 300 CFU /ml (Elizabeth et al., 2005). Time-kill curve data were plotted as logarithmic declines in CFU per milliliter versus time. The curves were evaluated by visual inspection for time to a 3 log₁₀ unit decline in bacterial numbers and total logarithmic decline in bacterial numbers. Because of small sample sizes, inferential statistics were not applied to these data. Time kill curves were constructed by plotting the log CFU/ml versus time in hours.

RESULTS

Susceptibility testing

The pre exposure MIC of metronidazole for the *E. coli* isolate was >64 mg/L.

Time kill curves

The time kill curve for metronidazole with an MIC of >63 mg/L against *E. coli* was strikingly similar to the curve for the growth control. Activated charcoal achieved a 1log killing of *E. coli*, and this was doubled in the presence of metronidazole - AC of note, significant re-growth occurs with AC alone following a reduction of the starting inoculums to below the LLA after 3 h (Figure 1), thus reflecting adsorption. Metronidazole - AC produced very rapid 2log killing within the first one hour of the experiment as reflected in Figure 1. The decrease in the number of CFU/ml of *E. coli* by AC alone in the model was significantly greater than with metronidazole alone which had no activity against *E. coli* however, there was significant difference in the number of CFU/ml of *E. coli* in

Table 1. Concentration of AC and metronidazole- AC used for test tube analysis.

Test tube number	Volume of <i>E. coli</i> suspension containing 100 cfu/ml (ml)	Resultant concentration of AC in each test tube (ml)	Resultant concentration of metronidazole- AC in each test tube(µg/ml:mg/ml)
1	1	0.04	1:2
2	1	0.08	2:4
3	1	0.12	3:6
4	1	0.16	4:8
5	1	0.20	5:10
6	1	0.24	6:12
7	1	0.28	7:14
8	1	0.32	8:16
9	1	0.36	9:18
10	1	0.40	10:20
11	1	-	Control

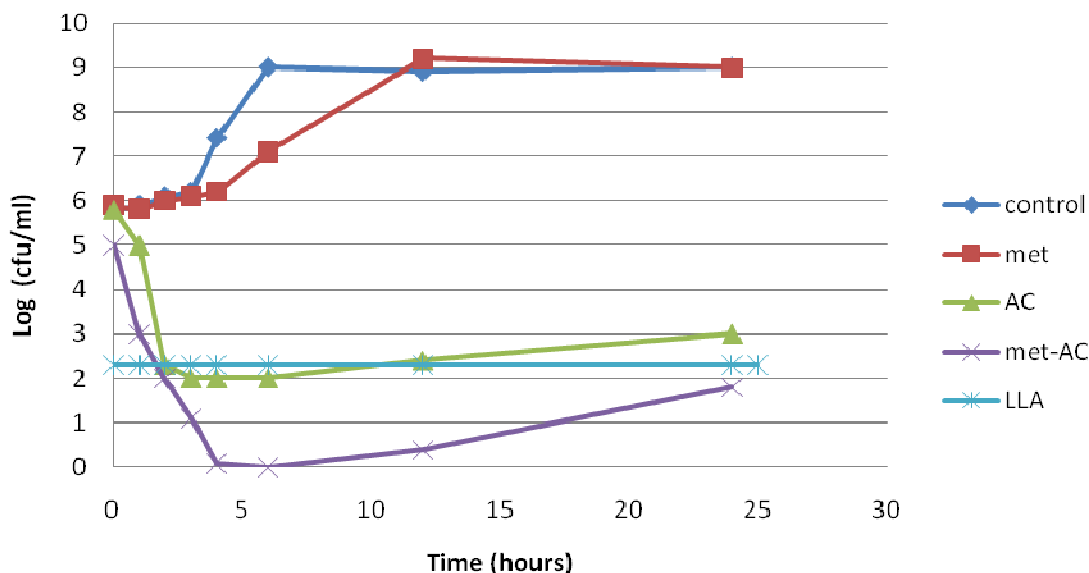


Figure 1. Combined time kill curve against *E. coli* 0157:H7 from exposure to metronidazole, activated charcoal and metronidazole-AC. met, metronidazole; AC, activated charcoal; met-AC, metronidazole-AC.

the metronidazole-AC model and there was no significant re-growth at 5.5 h (that is, absence of increase in the number of bacteria to quantifiable level following a 3log killing) compared to the model that had AC alone.

To determine the rate of killing, points were used for the determination of the slope until re-growth occurred. However, for the values of metronidazole-AC, the rate of killing was rapid that the log CFU/ml was below this study's LLA by 2 h. Thus, the slope was obtained as zero at the 2 h time point. The coefficient of determination in the linear regression analysis were greater than 0.91 except that for metronidazole against *E.coli* ($r^2 = 0.21$) which had a non-linear time kill curve. The absence of re-growth thereby extrapolated to the rate of killing was

significant in the metronidazole - AC combination.

Test tube analysis

The analysis in the test tubes however showed the highly adsorptive potential of activated charcoal. The tubes containing activated charcoal alone exhibited varying degrees of turbidity however at high concentrations of AC, 18 and 20 mg/ml, the plates incubated for 24 h showed no growth. However the tubes containing metronidazole - AC exhibited a reduced turbidity and plates incubated for 24 h from these tubes had no colony forming unit from concentrations of 5 µg/ml of metronidazole and

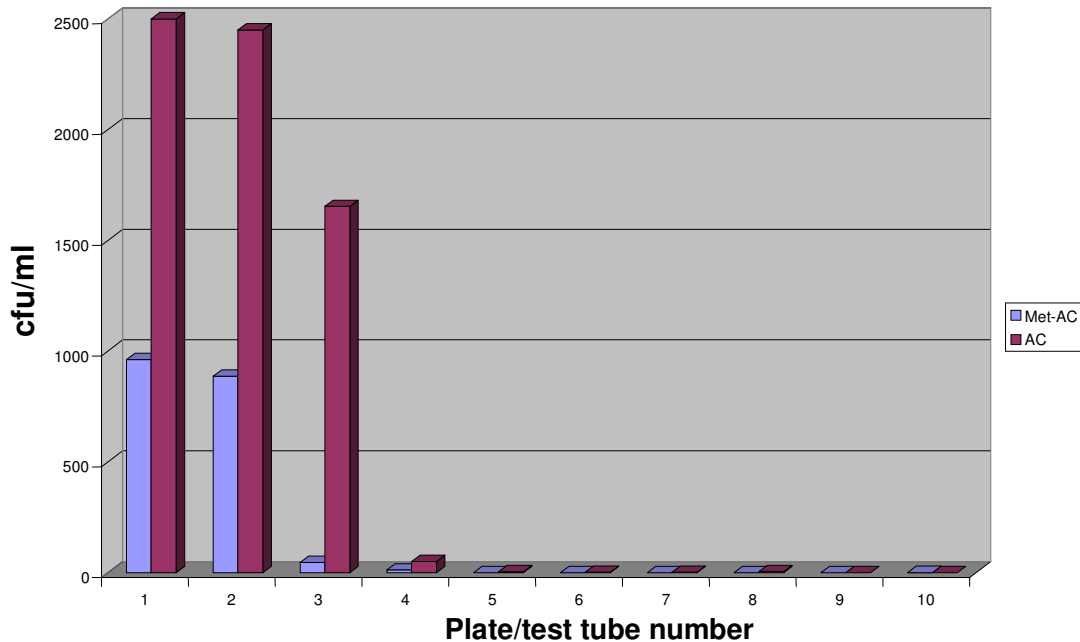


Figure 2. Comparison of the number of cfu/ml counted in the plates containing metronidazole- AC and AC alone.

10 mg/ml of activated charcoal.

DISCUSSION

This study shows the activity of metronidazole and activated charcoal given daily in an *in vitro* pharmacodynamic model utilizing *E. coli* 0157:H7, a diarrhea causative organism. *E. coli* 0157:H7 infection occurs via fecal oral route or by ingestion of foods especially beef which has been contaminated with the bacteria (Dahiru, 2008). The *E. coli* isolate, EHEC strain which causes a virulent form of diarrhea was utilized in this study because it has a higher MIC compared to other strains and this allowed visualization of the difference in the time-kill profiles which also reflects the adsorptive capacities of AC. The use of a less susceptible isolate represents a bad case scenario and thus provides a realistic estimate of the adsorptive capacity of AC alone and metronidazole-AC against *E. coli* which may be typically seen in diarrhea disease. In the presence of mixed infections, the anaerobic bacteria will be susceptible to metronidazole and possible aerobic bacteria like *E. coli* will also be adsorbed onto the metronidazole-AC interstices.

From Figure 1, the time kill curve (reflecting adsorption) for the combination of metronidazole-AC suggest that even though metronidazole has no activity against *E. coli* in the presence of AC, some synergistic activity was observed. The time kill curve also reflects the adsorptive ability of the activated charcoal in the pharmacodynamic

system. The adsorptive function of AC in Figure 2 appears to be concentration dependent, increasing linearly with increased AC concentration as reflected by decreased cfu/ml values. Where regrowth (Figure 1) was observed in the use of AC alone, the microorganism was recovered from the AC interstices via washing with sterile water and plating. However in the system where metronidazole-AC was utilized, recovery of *E. coli* was not successful as regrowth was not observed. It is postulated that the adsorption of metronidazole to the surface of AC as well as the micro-organism occurs at the same binding site. The presence of metronidazole-AC for some reason facilitates a weakening in the cell wall of the bacteria via an unknown mechanism which facilitates an adsorptive process reflected in Figure 1 as log killing. Recovery of micro organism from AC interstices serves as a pointer to assume that a “spread” (Dale et al., 2002), that is, indicating that adsorption on the surface of adsorbent AC occurs giving rise to the spreading pressure of the micro organism on the AC surface which increases incursion of the antimicrobial into the organism surface leading to a cell death. The biochemical process which follows this in an aerobic organism may yet fall into unknown mechanism of action category of texts (American Hospital Formulary Service, 2000). When AC is utilized alone, re-growth of organism occurred after a ten hour period and from Figure 1 it will be observed that at this point the log cfu/ml has risen above the LLA. In the presence of metronidazole –AC, the curve barely reaches the LLA after 24 h and this could be compared to the result from Figure 2 where at low concentration, no growth

is recorded at test tube 4 compared to that of AC alone; this is an indication of time and concentration dependent synergistic interaction. The area under the concentration time curve versus the MIC ratio for *E. coli* was 105 for metronidazole -AC and 26 for AC alone. The metronidazole -AC MIC for *E.coli* increased 5 fold when compared to utilization of AC alone or metronidazole alone. The standard dose of metronidazole, 400 to 100 mg every 8 h (American Hospital Formulary Service, 2000) was not used in the pharmacodynamic model, however metronidazole having concentration dependent bacterial activity and a favorably safety profile allowed us to utilize a dose which can be replicated and results obtained at higher doses as well as more frequent time intervals.

Conclusion

The use of metronidazole-AC caused a significant reduction in the concentration of starting inoculums of *E. coli* 0157:H7 via a significant 3-log killing below LLA. Variation in MIC data of metronidazole was altered in the presence of AC. The use of metronidazole- AC will be useful in the treatment of diarrhea caused by *E. coli* 0157:H7. Further studies utilizing appropriate animal model is required and a further extrapolation to human model done.

ACKNOWLEDGMENT

The author acknowledges Mr. Usman of the Department of Pharmaceutical Microbiology of the University of Lagos for his advice on the design of the *in vitro* dynamic model.

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