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Full Length Research Paper

Effect of delayed sterilization on the production of intravenous fluids (parenterals)

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The effect of delayed sterilization on the production of intravenous fluids using 5% (w/v) dextrose solution was studied. The 5% (w/v) dextrose was prepared with distilled water. The solution was inoculated with a laboratory isolate of *Escherichia coli* and thereafter divided into two: Solutions A and B. Sample A was sterilized within 1 h of innoculation, while B was incubated at 37°C for 48 h and sterilized after. The microbial load was determined before sterilization. Both samples were then tested for pyrogenicity using rabbit test of pyrogen. The results revealed a significant ($P < 0.05$) increase in *E. coli* count in solution B from 8 ± 2 to 99 ± 5 cfu/ml after 48 h. Sample A became pyrogen-free after sterilization (sum of response (SR) of 3 rabbits, 0.75°C), while sample B contained 40 EU/ml (limit: ≤ 0.5 EU/ml for large volume parenteral). A 10 times dilution of sample B with pyrogen-free 0.9% w/v sodium chloride produced a response of 2.80°C and SR of 3 rabbits (failed limit ≥ 2.65°C). From this study, a delay of up to 48 h before sterilizing solutions intended for parenteral use could produce high pyrogenicity.

Key words: Parenterals, pyrogen, *Escherichia coli*, sterilization, microbial load.

INTRODUCTION

Intravenuous fluids are medications usually in volumes greater than 100 ml and are called large volume parenterals. The fluids which are administered through the veins must be sterile, particle and pyrogen-free, as well as must carry labels containing the constitutents for normal fluid as per the label claims (British Pharmacopoeia, 2002).

Large volume parenterals are occassionally found contaminated with fungi, pathogenic bacteria and pyrogens (which are bacterial endotoxin) (Atata et al., 2007). Systemic injection of these pyrogens causes a cascade of reactions leading to the release of cytokines by macrophages and monocytes. The resultant effect include elevated body temperature and other adverse reactions characteristic of sepsis such as systemic inflammatory response syndrome and infection (Bone et al., 1992). Exposure to pyrogens have been reported to have adverse effect such as hypotension, decreased cardiac output, increased pulmonary arterial pressure and vascular permeability in the lungs. Others include disseminated intravascular coagulation, activation and sequential damage to the heart, liver and lungs known as "Multiple Organ Failure Syndrome" (Charles, 2000). Therefore, there is the need to continue to make intravenous fluid that are free of pyrogens. Manufacturers of intravenous fluids are sometimes sanctioned for violation of current Good Manufacturing Practices (GMP) especially when cases of contamination of intravenous fluids are established. Contamination can result from poor and unhygienic handling during production (Hippenmier, 1978) or in transit as well as during storage.

The possible causes for the high pyrogen content of some infusions found in the Nigerian market had been attributed to non-compliance with GMP by the National Agency for Food and Drug Administration and Control

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Abbreviations: SDA, Sabouraud dextrose agar; **EMB,** eosin-methylene blue agar; **NA,** nutrient agar; **GMP,** good manufacturing practices; **CFU,** colony forming unit; **TNTC,** too numerous to count.

(NAFDAC). However, there is need to investigate specific factor likely to account for such high pyrogen content of sterile preparations. In a country like Nigeria where supply of electricity is epileptic, manufacturers are faced with disruption in batch processing which leads to a possible delay in completion of a cycle of production. Manufacturers of intravenous fluids are not exempted from this.

The objective of this study was to establish a link between the duration of production cycle and the pyrogen content of the resultant fluid. We also attempted to produce endotoxin from *Escherichia coli* using dextrose 5% (w/v) as medium with a view to producing laboratory method for the preparation of bacterial endotoxin.

The production of an infusion was simulated and different times of final sterilization of the solutions contaminated with *E. coli* were imposed. The resultant solutions were tested for initial microbial load and microbial load before sterilization and post sterilization. Pyrogen levels of the fluids obtained after sterilizations were tested using the official testing method for pyrogen according to The British Pharmacopoeia (2002).

MATERIALS AND METHODS

Materials

Pharmaceutical grade dextrose monohydrate, normally used for production of infusion was a product of Roquette Pharma, Cedex, Lestrem, France. *E.coli* was obtained from the Quality Control Laboratory of Biomedical Limited, Ilorin, Nigeria. Pyrogen-free, sterile, distilled water was used as solvent. Albino rabbits were obtained from the Animal House of Biomedical Limited, Ilorin, Nigeria. Eosin methelene blue (EMB) agar, nutrient agar (NA) and sarboraud dextrose agar (SDA) were products of Sigma Aldrich Co Ltd., Poole Dorset, UK.

Procedures

The production of dextrose solution (5% w/v) infusion was simulated using pyrogen-free distilled water. Actual infusion from pharmacies were not used because the condition of preparation could not be controlled for the purpose of this investigation.

The solution was inoculated with laboratory isolates of *E. coli*. The microbial load of the solution was estimated by plating 1 ml of the sample on agar plates of SDA, EMB and nutrient agar (NA). Five replicates were made for each agar plate. EMB and NA plates were thereafter incubated at 37°C for 24 h, while the SDA plates were incubated for 48 h. This represent the initial microbial load of all subsequent samples. The remaining solution was divided into two equal portions, A and B, and kept in sterile, de-pyrogenated (by heating in an oven at 250°C for 3 h) glass conical flasks (European Pharmacopoeia 2000).

Soution A which is the control was autoclaved at 118°C, 1.5 atm for 35 min within 1 h after the inoculation with *E. coli*; while solution B was incubated at 37°C for 48 h. At the end of 48 h, sample B was estimated for microbial load and treated as described earlier. The solution B was later autoclaved at 118°C, 1.5 atm. for 35 min. Solutions B were thereafter subjected to microbial load estimation to obtain the final microbial load before sterilization and was done again after sterilization.

The sterilized solutiions A and B were subjected to pyrogen tests using the Rabbit test method (B. P., 2002). Briefly, sample A was injected into a set of three rabbits at a dose of 10 ml/kg, while sample B was diluted 1:9 with pyrogen-free, sterile 0.9% w/v sodium chloride solution to reduce the expected endotoxin concentration by a factor of 10. Another dilution, 1:99 was done with the same diluent to give 100 percent diluted pyrogen content. Each of these solutions were injected into a set of 3 rabbits at a dose of 10 ml/kg.

A control group was set up in parallel with the test groups by injecting sterile and pyrogen-free 0.9% w/v NaCl into another set of three rabbits as earlier described (B. P. 2002). The sum of response of 3 rabbits was determined.

Statistical analysis

The students t test was used. Results are expressed as mean ± SEM of 5 determinations.

RESULTS

The results in Table 1 shows that there was significant difference between initial microbial load and microbial load 48 h after innoculation ($p < 0.05$). The final post-sterilization microbial load was nil, meaning that the product could be sterile. The results on the pyrogen tests were interpreted according to the standard official method used. The pyrogen test result is as shown in Table 2. The sample B could not be injected directly in anticipation of a high pyrogen content but had to be diluted 1:9 and 1:99.

The initial bacterial loads of A and B were actually the same. However, after 48 h, sample A had zero count, while B had an *E.coli* population of 99 ± 5 cfu/ml. The bacterial count of sample B was too numerous to count (TNTC) after 48 h, meaning that bacteria cells have greatly multiplied during the time lag. Fungal count increased from 1 ± 0.5 to 10 ± 1 cfu/ml (Table 1). These final counts were significantly different ($P < 0.05$). The pyrogen test results as shown in Table 2 indicated that sample A that was sterilized within 1 h after contamination with *E.coli* became pyrogen free (sum of response of 3 rabbits, 0.75°C), while sample B sterilized in 48 h after contamination with *E.coli* failed pyrogen test when diluted 10 times with pyrogen-free saline solution (Table 2).

DISCUSSION

Manufacturers of infusions like any other manufacturer will not deliberately delay a production process such as sterilization, but local problem of electricity supply or any other technical hitch could cause a delay. Deliberate contamination cannot also happen. However, in the case of accidental contamination of the fluid, between mixing and sterilization, maybe due to human contact while filling, with a Gram negative pathogen such as *E. coli* could be dangerous.

From our findings, a delay of up to 48 h before sterilization produced a sterile but pyrogenic solution. An average response of 0.93°C was observed for the 1:9

Table 1. Microbial load of *E.coli* contaminated dextrose 5% w/v solutions.

Medium	Initial count (cfu/ml)		Count (after 48 hrs)		Final count (after autoclaving)	
	A	B	A	B	A	B
SDA (fungi/moulds)	1 ± 0.5^{a}	1 ± 0.5^{a}	n.a.	10 ± 1^{ab}	Nil	Nil
EMB (E.coli)	8 ± 2^{c}	8 ± 2^{c}	n.a.	99 ± 5^{cd}	Nil	Nil
NA (bacteria)	10 ± 2^{e}	10 ± 2^{e}	n.a..	$TNTC^{ef}$	Nil	Nil

Values are expressed in mean ± SEM of 5 determinations. Values carrying diferent superscripts are significantly different at $P < 0.05$. TNTC = Too numerous to count; n.a. = not applicable.

Table 2. Pyrogen Test Results of E.coli contaminated 5% Dextrose solutions.

Sample	Sum of response of 3 rabbits (°C)	Pass limits (°C)	Fail limit (°C)	Comments
Control (0.9%, w/v NaCl)	0.20	≤1.15	≥2.65	Pyrogen-free
A	0.75	≤1.15	≥2.65	Pyrogen-free
B (1:99 in 0.9%, w/v NaCl)	1.35	≤1.15	≥2.65	Slightly pyrogenic
B (1:9 in 0.9%, w/v NaCl)	2.80	≤1.15	≥2.65	Highly pyrogenic

Results interpreted according to the British Pharmacopoeia, 2002.
Sum of response of 3 rabbits measured as rise in body temperature over a period of 3 h.
A pyrogen free solution should produce a maximum of 1.15 sum of rise in temperature.

diluted sample. According to Dalmora et al. (2004), the equivalent amount of endotoxin in the solution injected which is capable of eliciting such reaction is equivalent to 4.0 EU/ml/kg body weight. The animals received 10 ml/kg solution that was diluted 10 times with pyrogen free physiological saline. Then the estimated content of the solution of pyrogen is therefore 40 EU/ml. This is highly toxic. The upper limit of bacterial endotoxin in large volume parenterals is 0.5 EU/ml. Sample A that was sterilized within 1 h after innoculation became pyrogen-free after sterilization. This result indicates that delay in serilization further increases the risk of high pyrogen content of large volume parenterals (Table 2). Therefore, manufactures could be advised to make small batches that could be quickly filled and sterilized within reasonable time as prolonged time before sterilization will lead to proliferation of contaminating microbes and possibly high pyrogenicity.

In this study, dextrose solution in water has proved to be a supporting medium for the growth of *E. coli* which has been the source of the pyrogen (Table 2) as a result of increased microbial load of gram negative pathogens when present as a contaminant (Table 1).

Intravenuous fluids should be manufactured under controlled environments. The processes of manufacture should be carried out under various classes of air with respect to total viable count of organisms per cubic metre depending on the type of activity carried out in a compartment. The critical processes should be carried out by highly trained personnel under asceptic conditions. Minimal human contact with the product during compounding, filling and stoppering and before sterilization is required. Terminal sterilization is required for parenterals which are not thermolabile, to remove the possible minimal contamination of the bulk product during processing. Hence sterilization should be done within a very short period after a solution have been prepared.

Conclusion

This research has conclusively investigated a possible explanation to the high pyrogenicity of infusions that are though sterile but pyrogenic. It underscores the contribution of lack of infrastuctures such as electricity to the processing of sensitive products such as infusions and its high risk to the Nigerian economy.

In this research, endotoxin was successfully prepared using a simple culture of dextrose 5% (w/v) solution contaminated with *E. coli*. The potency of the endotoxin (pyrogen) is so high that the diluted samples still caused fever in the test animals in unacceptable levels.

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