A cell viability assay to determine the cytotoxic effects of water contaminated by microbes

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Many South Africans do not have access to safe drinking water, so they have no alternative but to use water from contaminated sources that poses a health hazard. This poor state of affairs appears to be deteriorating. In order to distinguish safe from unsafe sources, the aim of this study was to adapt the well-known MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay into a simple and efficient method to screen the suitability of drinking water without needing to know the nature of any possible contamination. This modified assay presents an immediate and reliable answer to whether water is potable without recourse to standard chemical and microbiological water-quality tests. The MTT assay was used here for the first time to test the effects of microbes, and not chemical contaminants as is traditionally the case, on the viability of human duodenum cells exposed to water samples of interest. Filtered tap water and water from a borehole, for example, had limited adverse effects on cell viability. Cell viability decreased greatly after exposure to dam, treated sewage and river water which confirmed the value of the assay as a screening tool.

Introduction

A common problem in rural areas of developing countries, such as South Africa, is the poor quality of drinking water from some available sources. Rural communities often do not have access to treated drinking water and they have no alternative but to use water directly from untreated sources such as streams, rivers, wells and ponds.[1] Water quality is most commonly accepted as potable when its chemical properties lie within the limits of what is regarded as safe, and when microbiological analysis confirms the absence of harmful agents.[2–4]

Contamination of water by microbes means that an increasing number of rural communities reliant on untreated sources are exposed to water-related diseases.[5] These diseases include shigellosis, cholera, salmonellosis, diarrhoea and a variety of viral, bacterial, fungal and protozoan infections.[6] People who are particularly susceptible to these diseases include those with underdeveloped, compromised or weakened immune systems, such as, respectively, very young children, individuals living with HIV/AIDS and the elderly.[7] It is desirable to screen drinking water, before susceptible people are exposed to it, with a single, quick and effective method, using a live model without the ethical implications of involving laboratory animals. The abiotic parameters of water quality such as pH, electrical conductivity and dissolved oxygen are indicators that overlook their biological impact.

The aim of the study reported here was to develop a relatively quick, inexpensive and effective method with which the safety of water for human consumption can be gauged. The main criterion for such an assay was that it should indicate whether the water might be harmful to humans. The assay need not distinguish among possible contaminants, whether chemical or microbial, but must present a result at least partially based on the response of the human body. For this reason an intestinal mammalian cell line was selected as the test tissue culture. The HuTu-80 cell line (human duodenum adenocarcinoma) (hereafter referred to simply as 'cells') was chosen as a model for the human intestine to indicate the cytotoxic effects of microbes in untreated drinking water.

The viability of the cells was determined by the MTT assay, which is a colorimetric method based on the metabolic ability of the cells to reduce the yellow tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to a blue crystalline formazan product.[8] The number of viable cells after exposure is directly proportional to the amount of formazan produced, which can be quantified spectrophotometrically.[9] This method is widely used to analyse the cytotoxicity, cell viability and proliferation of living cells in the 96-well plate format,[10] usually to determine the effect of chemical pollutants[11,12] or biological proteins[13,14]. It is not used to determine the effect of microbes, which can also metabolise the MTT, thereby obscuring their detrimental effect on the cells.

However, our assay needed also to consider microbial contamination. Of the main categories of contamination (microbial and chemical), it would be the microbial contaminants that would be the most likely to have adverse effects on cell viability. The levels of chemical contaminants in the natural environment cause either acute toxicity (at high levels) or chronic toxicity (at low levels).[15] When chemical contaminants occur at concentrations high enough to cause acute toxicity, fish and other biota would be dead or dying and people would be less likely to consume water from such a source. Low chemical concentrations rarely have an effect on cell viability unless a water sample is extracted for the target pollutants and concentrated many times.[16] Based on these assumptions, we adapted the well-known MTT assay in such a way as to screen for microbial contaminants. However, the possible effect of chemicals on cell viability was controlled for in one instance, which is discussed in the Methods section.

Methods

Maintenance of cells

HuTu-80 cells (American Type Culture Collection (ATCC): HTB-40™) were obtained from the ATCC (Manassas, VA, USA). They were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma D2902, Schnelldorf, Germany) at pH 7.1 and supplemented with 10% foetal bovine serum (Thermo Scientific Hyclone, Cramlington, UK) and 0.08 mol NaHCO$_3$. The cells were grown in tissue culture dishes (90 mm x 20 mm) (Tooltech Pty, Johannesburg,

South Africa) in a humidified atmosphere at 37 °C and 5% CO_2 with media changes every 2–3 days.[17] Cells were passaged when confluent.

Water samples

The sampling points were all in and around Potchefstroom, a town in the North West Province of South Africa. Water expected to contain different microbial loads was sampled from Potchefstroom Dam, Mooi River, treated sewage from the municipal wastewater treatment plant, the municipal purification plant (tap water) and a borehole. Approximately 1 L of water from each of the five sources was collected in sterilised glass bottles and protected from ultraviolet radiation during transportation to the laboratory. The water samples were stored at 4 °C until tested within 24 h of collection.

The negative influence on human health of disinfection by-products of water purification chemicals has been reported previously.[18] In order to distinguish between the possible detrimental effects of the chemicals and microbes that might be present in the tap water, the water was filtered through a 0.22-μm filter to remove any microbes present. Any impact on viability caused by the filtered tap water would be attributed to the disinfection by-products. The cells were exposed to the different water types by dissolving the DMEM powder directly into the sampled water.

Control media and test media containing
the microbial exudates

The pathological effects of microbes result from their exudates, which may include enzymes such as proteinases, DNases, lipases, hyaluronidases, chondroitinases and lecithinases that affect the cells' metabolism to such an extent that they die. The microbes secrete these exudates only when in physical contact with mammalian cells.[19] Because microbes can also metabolise MTT, which would adversely affect the results of the assay, the challenge was to use the MTT assay to measure the effects of the microbial exudates on cell viability but in the absence of the microbes themselves. To achieve this, the test media were prepared by exposing cells to the water samples for 16 h. These initial exposures were conducted in tissue culture dishes with the same cell densities, and with cells of the same origin and history. The test media were harvested and sequentially filtered through 1-μm, 0.45-μm and 0.22-μm bottle-top filters (Corning, Lowell, IN, USA) to remove all the microbes and cell

debris. Sterile media were prepared as a control for the nutrient-depleted media's effects on cell viability (referred to here as 'control media'). The control media were also harvested after 16 h and underwent the same filtration series to eliminate possible effects introduced by the filters.

MTT assay

A new set of cells was exposed to the test media (containing microbial exudates) and control media (excluding exudates) in a 96-well microtitre plate (Greiner Bio-One, Frickenhausen, Germany). The cells were seeded at a density of 20 000 cells per well and incubated for 16 h to adhere to the bottom of the plate.[20,21] In a previous study using a Roche real-time cell analyser (xCELLigence system),[22] this seeding density was found to be the most effective for the exposure period (results not shown). The xCELLigence system measures electrical impedance across microelectrodes on the bottom of each well in a plate. On each plate 12 wells received freshly prepared sterile media. Six of these represented positive control cells (assumes maximum viability). The other six were killed with absolute methanol at the end of each experiment (assumes negative control cells, minimum viability). The different test media, control media, and positive and negative controls were separated by a row/column of wells filled with phosphate-buffered saline to avoid cross-contamination. Cells were exposed for 6-h and 12-h periods. There were two plates per water type, one for each exposure period. The number of replicates for each type of medium and cell control is indicated in Table 1. At the end of the exposure period the medium of each well was replaced by 0.5 mg/mL MTT (Sigma M5655) and incubated at 37 °C for 30 min. The MTT solution was replaced with dimethyl sulphoxide (Merck, Darmstadt, Germany) to dissolve the newly formed formazan crystals. Absorbance of exposed test media, control media and positive control media was measured at 540 nm.[23] The optical density of the solubilised formazan is directly proportional to the number of viable cells per well.[24,25] Viability was calculated by expressing the absorbance of exposed test media and control media as a percentage of the absorbance of the positive control cells. The viability of the test media was further expressed as a percentage of the control media for each plate (Table 1). This calculation indicates the effect of the water type only on viability, excluding the possible effects of used media or unknown inherent variables.

Table 1: Cell viability after exposure to different water samples (test media) and corresponding control media

Water samples	Exposure period (h)	Test media (TM) (% viability)	Control media (CM) (% viability)	TM/CM x 100	Survival index
Positive control	6	100 ± 15.1	100 ± 15.1	100	1.0
	12	100 ± 17.7	100 ± 17.7	100	1.0
Filtered tap	6	93.7 ± 11	85.4 ± 13.8	109	1.1
	12	77.3 ± 12	65.3 ± 9.2	118	1.2
Borehole	6	79.6 ± 14.7	78.3 ± 12.1	101	1.0
	12	78.5 ± 11.1	75.5 ± 16.1	104	1.0
Unfiltered tap	6	74.2 ± 10.9	88 ± 8.8	84	0.8
	12	60.6 ± 10.5	88.5 ± 13.9	68	0.7
River	6	-1.9 ± 1.2 ≈ 0	101.1 ± 15.2	0	0
	12	-4.8 ± 0.7 ≈ 0	71.9 ± 11.5	0	0
Treated sewage	6	-3.4 ± 1.4 ≈ 0	103.5 ± 12.5	0	0
	12	-6.9 ± 1.2 ≈ 0	91.5 ± 21.6	0	0
Dam	6	-6.9 ± 0.9 ≈ 0	104.2 ± 14.4	0	0
	12	-8.7 ± 0.7 ≈ 0	85.5 ± 22.8	0	0
Negative control	6	0 ± 0.04	0 ± 0.04	0	0
	12	0 ± 0.02	0 ± 0.02	0	0

Note: n=18 except for positive and negative controls where n=6; negative values were rounded to 0 because all cells were dead and therefore had 0% viability.

Survival index

The outcomes of the assay were also transformed into a survival index by dividing the values in the 5th column of Table 1 by the viability of the positive control, which is 100%. The survival index indicates the response of the cells in relation to the positive control: a survival index greater than 1 indicates a better survival and a value less than 1 represents definite cytotoxic effects (Table 1).

Statistical analysis

SPSS software[26] was used for all statistical tests, except the Box-Cox analyses that were performed in Statistica[27]. The data were investigated for normality (Shapiro-Wilk) and subjected to Box-Cox analysis to select an appropriate logarithmic transformation to improve normality in those instances where the data were not normally distributed. The means of the transformed data were compared using one-way analysis of variance with Tukey HSD post-hoc tests performed on data with equal variances and with the Games-Howell test on data with unequal variances. Levene's test was used to determine the equality of variances. The values in Table 1 were back-calculated from the transformed data to meaningful values.

Results and discussion

The effect on the cells after exposure to river, treated sewage and dam water was particularly severe; the entire population of exposed cells was destroyed even after only 6 h of exposure (Table 1). The difference in means between the test media and the corresponding control media was statistically significant for both exposure periods ($p < 0.05$). For the remaining types of water, the viability decreased significantly ($p < 0.05$) as follows: filtered tap > borehole > unfiltered tap water. This pattern was evident for both exposure periods (Table 1).

When exposure periods were compared, for the tap water (both filtered and unfiltered) the difference in viability was significant ($p < 0.05$). For the filtered tap water, the longer exposure led to better survival than did the shorter, in contrast to the unfiltered tap water, in which case the cells exposed for 6 h survived better than those exposed for 12 h (Table 1). There was no significant difference in the viability of cells exposed to the borehole water for the two exposure periods.

The MTT test, as applied in this study, was successful in discriminating between potable water and water that is probably not safe for human consumption. The test results clearly indicated that the dam, treated sewage and river water caused adverse effects on the intestinal cells' viability. Microscopic investigation of the exposed cells showed them decaying and microbial activity increasing with exposure time (results not shown), indicating that the decrease in cell viability was as a result of microbial activity rather than possible chemicals in the sampled water.

For water types that allowed the cells to survive — that is, filtered and unfiltered tap water and the borehole water — the assay could distinguish degrees of survival. Filtered tap water had a higher survival success (indicated by a higher survival index in Table 1) than the unfiltered tap water, indicating possible microbial activity in the tap water. The tap water was filtered to investigate the possible detrimental effects of the added purification chemicals, but from these results the chemicals do not seem to have influenced cell viability. Contrary to what was expected, the cells in the filtered tap water survived even better than those in the positive control (Table 1). This finding may be a result of the additional nutrients available in the tap water that were not available in the water of the positive control, which was double distilled and autoclaved.

The slight detrimental effect of tap water (Table 1) was unexpected as this was a typical example of the potable water quality of the local municipality, which has Blue Drop status.[28] Blue Drop status is awarded in an incentive-based regulatory scheme by which the Department of Water and Environmental Affairs of South Africa monitors the quality of potable water. Blue Drop status is awarded to any municipality that scores over 95% for all categories audited.[29] Although the unfiltered tap water supported above-average survival of the cells, the current water quality might not be conducive to people with compromised health (HIV/AIDS sufferers, people on chemotherapy, babies and the elderly).

Although the assay could differentiate between the shorter and longer exposure periods for filtered and unfiltered tap water, the reasons for the inconsistent pattern (12 h exposure > 6 h viability for filtered tap water but 12 h exposure < 6 h viability for unfiltered tap water) require further investigation. What is important is that the assay did distinguish between different exposure times when there was a difference to observe.

It seems as if the borehole water did not contain enough microbes at the time of sampling to influence cell viability significantly. This borehole is situated 17 km east-north-east of Potchefstroom, on a grain crop farm. The neighbouring farms have cattle. Loop Spruit that drains the West Rand gold mines[30] runs across the farm, but if any of the possible pollutants had found their way to the borehole, their concentrations were too low in the 1-L sample to have had a detrimental effect on the cells.

The above results showed that the assay is useful as a first tier screening of drinking water quality — it provides quick results and tests the water that is directly available to the consumer. It uses human intestinal cells as a model for humans, thereby giving a better indication of the possible health effects of the water than tissues from another mammalian or vertebrate surrogate would. No whole animal testing is involved, which avoids ethical considerations; and the potential high costs involved with maintaining laboratory animals are not an issue.

The assay can only be performed in a well-equipped tissue culture laboratory, which makes this test available to research laboratories and only to a few water-quality laboratories in South Africa. Because this assay does not measure any of the end points listed in the drinking water quality standards of the country,[2] there is no incentive for water monitoring laboratories to establish such a method. The applicability of this assay to test water quality over long periods of time, i.e. years, still needs to be proven. One of the biggest obstacles in this regard is finding a suitable internal reference for microbial load, so that the viability can be expressed in terms thereof. Results normalised in this fashion will enable comparison between samples obtained from various sites or at different times. The internal standard should ideally be one that determines pathogenic microbes, rather than all microbes.

A meaningful link between the laboratory results and their implication for human health (in the real world) needs to be established. A contributing factor that requires elucidation is the effect gastric fluids would have on swallowed water when it travels through the stomach to the duodenum. Gastric fluids might influence the microbial activity of the water.[31]

The survival index was developed as a tool for decision-makers to identify which water sources pose the greatest risk and which to prioritise for treatment.

Conclusions

We have demonstrated, for the first time, how an intestinal human cell line, Hutu-80, can be used as a model for the human digestive system and as the basis for a modified MTT assay to indicate the microbial content, specifically of drinking water.

The assay has a short turnaround time (results can be obtained within 36 h), is efficient and cost-effective, and can be used, in particular, to demonstrate whether water from a variety of sources is safe for human consumption. However, in interpreting results from such a cell-based assay, one should keep in mind that cells *in vitro* are more responsive than the same cells *in vivo* when they are protected by an immune system.

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Authors' contributions

S.P. carried out the research as part of her Master's dissertation and drafted the manuscript. R.P. supervised the work and assisted in the development of the concepts. C.C.B. co-supervised and reviewed the manuscript.

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