Effects of drinking desalinated seawater on cell viability and proliferation

Camila Longhi Macarrão, André Luis Lacerda Bachi, Mario Mariano and Lucia Jamli Abel

ABSTRACT

Desalination of seawater is becoming an important means to address the increasing scarcity of freshwater resources in the world. Seawater has been used as drinking water in the health, food, and medical fields and various beneficial effects have been suggested, although not confirmed. Given the presence of 63 minerals and trace elements in drinking desalinated seawater (63 DSW), we evaluated their effects on the behavior of tumorigenic and nontumorigenic cells through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay and annexin-V-fluorescein isothiocyanate/propidium iodide staining. Our results showed that cell viability and proliferation in the presence of 63 DSW were significantly greater than in mineral water and in the presence of fetal bovine serum in a dose-dependent manner. Furthermore, 63 DSW showed no toxic effect on murine embryonic fibroblast (NIH-3T3) and murine melanoma (B16-F10) cells. In another assay, we also showed that pre-treatment of non-adherent THP-1 cells with 63 DSW reduces apoptosis incidence, suggesting a protective effect against cell death. We conclude that cell viability and proliferation were improved by the mineral components of 63 DSW and this effect can guide further studies on health effects associated with DSW consumption.

Key words | apoptosis, cell proliferation, cell viability, cytotoxicity analysis, desalinated seawater

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ABBREVIATIONS

DSW	desalinated seawater
63 DSW	drinking desalinated seawater with 63 minerals
dSW	desalinated deep seawater
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MW	mineral water
PI	propidium iodide
RPMI-63 DSW	RPMI medium prepared with 63 DSW
RPMI-MW	RPMI medium prepared with MW
RO	reverse osmosis

doi: 10.2166/wh.2017.252

INTRODUCTION

Water desalination has been increasingly used to provide drinking water where freshwater is scarce. By 2025, it is estimated that more than three billion people will live under scarce water or water-stressed conditions (Seckler *et al.* 1999).

Almost 97% of water in the world is seawater and desalination technologies can be used to solve the freshwater crisis, to help meet demands for potable water, especially in coastal areas. Desalination plants operate in more than 120 countries in the world, including Saudi Arabia, Oman, United Arab Emirates, Spain, Cyprus, Malta, Gibraltar, Cape Verde, Portugal, Italy, India, China, Japan, and Australia (Darmon 1996).

Seawater contains more than 70 trace elements, including chemical components that contributed to the origin of life (Farrington 2000). However, the role of these trace elements and minerals is not completely defined. Desalination plants have used reverse osmosis (RO) technology to remove salt from seawater, making it drinkable (Greenlee *et al.* 2009). This process is already used in many countries where freshwater from rivers and lakes is scarce. Administration of desalinated deep seawater (dSW) has received much attention due to its beneficial effects on hyperlipidemia, atherosclerosis, and cardiovascular hemodynamics in animal model studies (Yoshioka *et al.* 2003; Miyamura *et al.* 2004). Furthermore, desalinated seawater (DSW) can promote an inhibitory effect on lipogenesis in the liver and might play an anti-obesity role by inhibiting adipocyte differentiation (Hwang *et al.* 2009).

In the health food and medical fields, dSW has been used as drinking water, and various beneficial effects have been suggested, although not scientifically confirmed. In particular, differences between surface and deep seawater were described, with potential application in the prevention of lifestyle-related disorders (Miyamura *et al.* 2004).

Other studies have described the benefits and therapeutic properties of dSW in the treatment of patients with several conditions, such as metastasis (Soyoung *et al.* 2013), eczema (Hataguchi *et al.* 2005), allergic rhinitis (Kimata *et al.* 2001), high blood pressure (Sheu *et al.* 2013), and diabetes (Ha *et al.* 2013). In Colombia, seawater has been used as a nutritional supplement since 2001 (Soler *et al.* 2006).

The aim of this study was to investigate the toxicity effects of drinking desalinated seawater with 63 minerals (63 DSW) and trace elements, on cellular viability, proliferation, and apoptosis incidence in murine embryonic fibroblast, murine melanoma, and non-adherent human cells.

METHODS

63 DSW

Drinking desalinated seawater 63 Water Vital Minerals® (63 DSW), which was provided by Ocean Par, Co., Ltd (São Paulo, SP, Brazil), was collected in the Atlantic Ocean (depth: 30 m) near the coast of Bertioga, SP, Brazil. The seawater was subjected to RO by which salt and impurities were removed and 63 elements of DSW remained, including major (B, Ca, Fe, K, Mg, Na, and S) and trace elements (e.g., Cu, Zn, and others). Bottled mineral water (MW), purchased at a local market, was used as a control. The main mineral components of drinking seawater are shown in Table 1.

Cell line and culture conditions

Tumorigenic (melanoma-derived B16-F10 cells, The Jackson Laboratory), nontumorigenic (NIH-3T3, murine embryonic fibroblast cells), and non-adherent cells (human acute monocytic leukemia THP-1 cell line; ATCC No. TIB-202) were maintained in RPMI-1640 medium (Sigma; St Louis, MO, USA) prepared with 63 DSW (RPMI-63 DSW) and mineral water (RPMI-MW), containing L-glutamine (2.0 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL), supplemented or not with fetal bovine serum (FBS; Cultilab) in 25 cm^2 culture flasks at 37 °C in a humidified atmosphere with 5% CO_2.

Cytotoxicity assay

Cell viability and proliferative assay were measured by Trypan blue exclusion method and reduction of

Table 1 | Major mineral contents in drinking desalinated seawater (63 DSW) and MW

Minerals	63 DSW (mg/L)	MW (mg/L)
Na	25.2	104
K	0.9	0.213
Mg	0.7	0.043
Ca	0.5	0.308
Cl	42.9	5.0
Zn	0.0068	NA

NA: not available.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide-MTT (Mosmann 1983; Al-Sheddi *et al.* 2014).

NIH-3T3 and B16-F10 cells in exponential growth stage were harvested from culture and resuspended in a fresh medium. The cell suspension was dispensed into 96-well culture microplates (Nunc, Corning Costar) containing 1×10^4 cell/well and incubated with RPMI-MW and RPMI-63 DSW with 10% FBS and in the presence of MW and 63 DSW and 63 DSW with FBS (25, 50, and 75%) during 48 h at 37 °C in a humidified atmosphere with 5% CO_2 and the cell viability was assessed by Trypan blue exclusion method.

In the cell proliferation assay, 1×10^4 cell/well obtained from NIH-3T3 and B16-F10 lines were cultured in RPMI-MW or RPMI-63 DSW with and without 10% FBS during 48 h at 37 °C. The MTT solution (20 μL) was added to each well and the cells were incubated for 2 h at 37 °C. The yellow tetrazolium salt was reduced to the dark-colored formazan by the viable cells. After the supernatant was removed, the formazan crystals were dissolved in isopropyl alcohol (100 μL) and the solution optical density (OD) was measured ($\lambda = 570$ nm) in an automated enzyme-linked immunosorbent assay reader (Beckman-Coulter Co., USA). The experiments were performed in triplicate to confirm the accuracy of the results.

Apoptosis assay

In order to determine the effect of 63 DSW on cell viability, we tested non-adherent cells (human acute monocytic leukemia THP-1 cell line) in the presence of RPMI complete medium (RPMI-MW or RPMI-63DSW) with 10% FBS. The THP-1 cells were fluorescently labeled by adding binding buffer (20 μL), annexin V-fluorescein isothiocyanate (FITC; 5 μL), and propidium iodide (PI; 5 μL). After incubation (in the dark for 15 min at room temperature), the cells were submitted to flow cytometry analysis. At least 10,000 cells in the gated region were analyzed in a flow cytometer (fluorescence-activated cell sorter (FACS) FACScan; Becton Dickinson; San Jose, CA, USA) using the CellQuest (BD) software. The results were interpreted using the percentage values of the total cells present in each quadrant. These experiments were independently repeated at least three times.

Statistical analysis

Differences between groups were analyzed using the two-sided *t* test and analysis of variance. All data were reported as mean ± standard deviation and $p < 0.05$ was considered statistically significant by the Graph Pad (Prism V. 4.00 for Windows) software.

RESULTS

The viability rates of murine embryonic fibroblast NIH-3T3 cells cultured in the presence of RPMI-MW or RPMI-63 DSW with 10% FBS were similar. The viability rates decreased in the presence of 63 DSW or MW, but were significantly higher in the presence of 63 DSW as compared to MW (Figure 1). As the 63 DSW or MW is not an appropriate medium for culture cells, the addition of FBS was a strategy to maintain the cells in culture during 48 h at 37 °C without the presence of RPMI. Therefore, the viability rates of murine embryonic fibroblast NIH-3T3 cells with 63 DSW were improved in the presence of FBS in a dose-dependent manner.

In the proliferation assay, a monolayer of NIH-3T3 cells was obtained after 48 h of incubation in RPMI medium prepared with MW and 63 DSW. The results showed that the proliferation rates of cells in the presence of RPMI-63 DSW were significantly higher than those in RPMI-MW with or without 10% FBS (Figure 2).

We also investigated the proliferative response of B16-F10 cells murine melanoma tumor cultured with RPMI-MW and RPMI-63 DSW supplemented with 10% FBS and compared to murine embryonic fibroblast NIH-3T3 cells. No difference was found in the presence of RPMI-MW supplemented with 10% FBS, but in the presence of RPMI-63 DSW with 10% FBS we observed the anti-proliferative effect in murine tumor B16-F10 cells when compared to murine embryonic fibroblast NIH-3T3 cells (Figure 3).

The effect of 63 DSW on apoptosis incidence in non-adherent THP-1 cells cultured with RPMI-MW and RPMI-63 DSW was analyzed by two-dimensional flow cytometry (FACS) using annexin-FITC + PI. The apoptosis rates in acute monocytic leukemia THP-1 cells cultured in RPMI-MW were higher than in the presence of RPMI-63

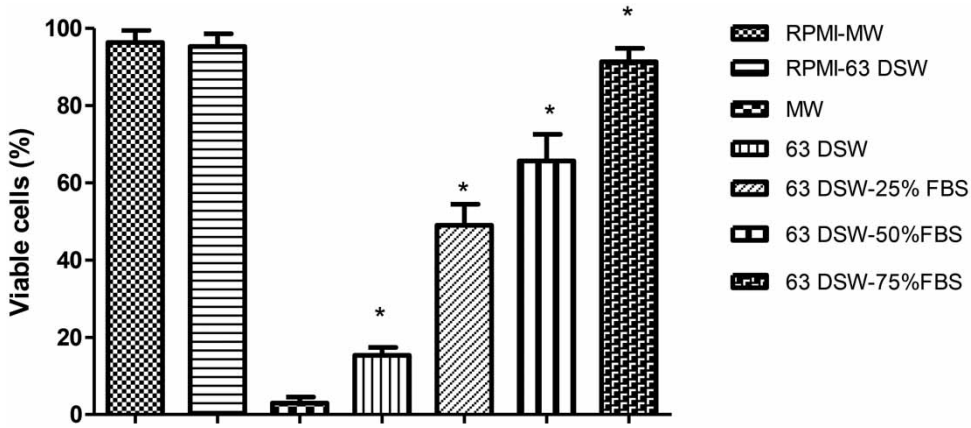

Figure 1 | Viability of murine embryonic fibroblast NIH-3T3 cells cultured in the presence of RPMI MW or RPMI 63 DSW with 10% FBS. The cells were incubated in the presence of MW and 63 DSW and different concentrations of FBS (25%, 50%, and 75%). Each column represents the mean ± standard deviation. *p values < 0.05 showed significant differences.

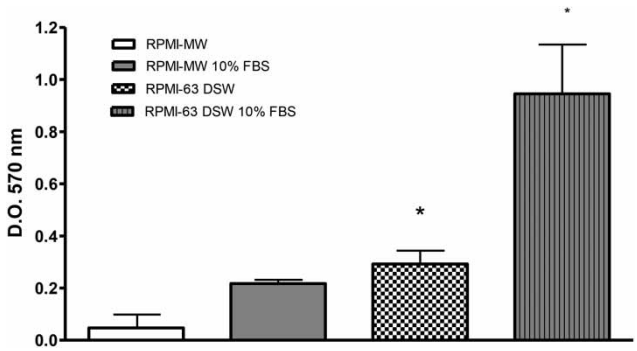

Figure 2 | Proliferation of murine embryonic fibroblast NIH-3T3 cells cultured in the presence of RPMI-MW and RPMI-63 DSW with or without addition of 10% FBS. Each column represents the mean ± standard deviation. Significant differences are indicated as * ($p < 0.05$).

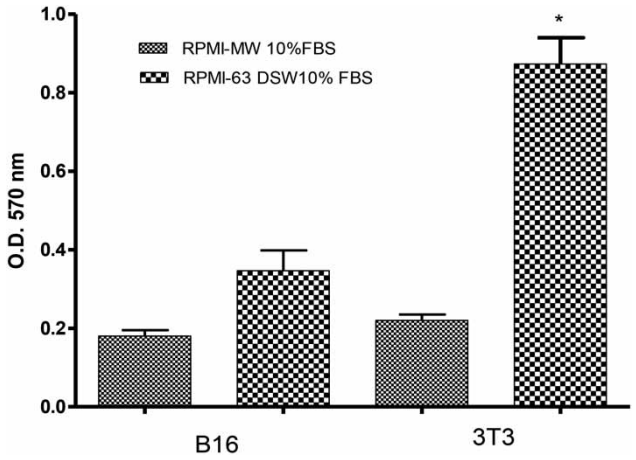

Figure 3 | Proliferation of B16-F10 and NIH-3T3 cells cultured in RPMI-MW and RPMI-63 DSW with 10% FBS as measured by optical density (DO). Significant differences are indicated as *$p < 0.05$.

DSW (90.6% vs 45.9%, respectively). The percentage of non-apoptotic cells cultured in RPMI-MW was lower than that in RPMI-63 DSW (0.215% vs 46.3%, respectively). In early apoptosis, the populations labeled with annexin V were similar (MW: 8.91% vs 63 DSW: 7.06%).

DISCUSSION

Desalination of sea and brackish water is widely practiced and is rapidly growing as the principal source of new freshwater in the world; however, the potential health effects of DSW consumption have not been fully investigated.

A variety of trace elements and minerals play an important role in body metabolism and are found in small amounts in most water sources (WHO 2000).

dSW is characteristically plentiful in nutrients rich in minerals such as Mg, Ca, K, and Zn, and its administration has been effective in the prevention of atherosclerosis (Miyamura *et al.* 2004), osteoporosis recovery (Liu *et al.* 2013), and in therapeutic intervention. Furthermore, it has drawn scientific interest for use in health food products, cosmetics, beverages, and agriculture (Hataguchi *et al.* 2005).

In other studies, the administration of dSW imbalance between loss and gain of essential minerals and trace elements underlies the delayed recovery from exhaustive physical challenge (Hou *et al.* 2013). Mg supplementation enhances exercise performance in gerbils (Wang *et al.*

2014) and dSW intake also can help prevent neointimal hyperplasia (or restenosis) (Li *et al.* 2014).

Our results showed that the viability rates of murine embryonic fibroblast (NIH-3T3) cells in the presence of 63 DSW were higher when compared to MW. Moreover, addition of FBS increased the cell viability and proliferation rates in a dose-dependent manner. In culture media, FBS is an important nutrient source contributing to 5–10% of composition, being able to influence the cellular replicative lifespan (Ryan 1979; Arigony *et al.* 2013). As shown in Figure 1, the cells were tested only in the presence of 63DSW and MW and addition of SFB, thus enabling the comparison of the effects of 63DSW and MW on the cells without the presence of RPMI medium.

In the cell proliferation assay, the index of cells in the presence of RPMI-63 DSW was higher than in RPMI-MW, suggesting that minerals and trace elements in 63 DSW are important for the viability of cultured cells. In fact, 63 DSW contains higher levels of the minerals Mg, Ca, K, Cl, Cu, and Zn than does MW (Table 1). Mg is an abundant divalent cation present in all living cells and acts as a cofactor in more than 300 enzymatic reactions (Lukaski 2000), cellular energy production, glycogen breakdown, and physiological regulation of neuromuscular functions (Bohl & Volpe 2002). Besides that, elevated Mg concentration stimulates human endothelial cell proliferation, which might translate into a beneficial effect in the setting of stent-associated vascular injury (Sternberg *et al.* 2012).

On the other hand, the reduction in intake of Mg and Ca due to the introduction of DSW in Israel and Qatar may be associated with potential increased risks for cardiac abnormalities, muscle cramps, hypertension, osteoporosis, and type 2 diabetes, due to a deficiency in these essential minerals (Spungen *et al.* 2013; Rowell *et al.* 2015).

Our results showed that the proliferation index of murine melanoma (B16-F10) cells was lower than that of murine embryonic fibroblast (NIH-3T3) cells in the presence of 63 DSW (Figure 3). We can suggest that the antitumor activity of 63 DSW may be derived from the combined ionic action of several minerals, such as calcium, magnesium, and potassium and trace elements in 63 DSW.

Magnesium deficiency is also known to be associated with the advancement of malignancy and metastasis and is frequently observed in patients with tumors (Kohli *et al.* 1989).

In another study, Soyoung *et al.* (2013) observed a reduction in the metastatic potential of breast cancer cells cultured in the presence of seawater. These authors suggested that seawater minerals can mediate biochemical reactions, signal pathways and cell migration in cancer patients, thus improving cancer survival by preventing tumor metastasis.

Apoptosis incidence in THP-1 cells cultured in suspension in the presence of 63 DSW was significantly reduced as compared to MW (Figure 4). We can conclude that synergistic action of the minerals and trace elements found in 63

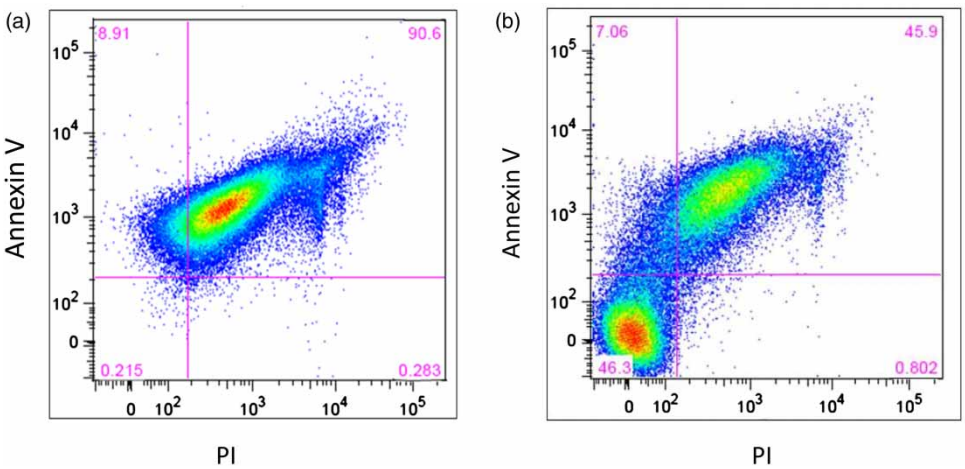

Figure 4 | Evaluation of apoptosis in non-adherent THP-1 cells cultured in RPMI-MW (a) and RPMI-63DSW (b), supplemented with 10% FBS. Cell apoptosis was determined by flow cytometry using annexin- FITC and PI.

DSW, in particular Mg and Zn, may be associated with antioxidant capability against oxidative stress eliminating free radical production and may have contributed to maintain cell viability.

In fact, trace elements in 63 DSW can participate in the antioxidant activity, which requires specific conditions such as medium, pH, temperature, oxidation state of the elements, and synergistic action of substrate. In addition, studies with cells in the presence of minerals and trace elements could generate indicators of cell and organism health (Van Gossum & Neve 1998).

The 63 DSW showed no toxic effect on murine embryonic fibroblast (NIH-3T3) and murine melanoma (B16-F10) cells. Preliminary results from our group have shown that the ingestion of 63 DSW in high doses and *ad libitum* in mice did not result in any signs of toxicity through hematological, biochemical, and histological analysis.

Our study revealed that the combined presence of 63 minerals including Ca and Mg and trace elements present in 63 DSW, promoted cell growth and higher viability as compared to MW.

CONCLUSION

We suggest that the presence of 63 minerals and trace elements in 63 DSW have influenced cultured cell performance in the viability and proliferation cell assays.

Studies in experimental animal models are important in order to understand the benefits and influence of minerals in 63 DSW on cell functions and health effects.

ACKNOWLEDGEMENTS

The authors are grateful to Dr Paulo Boschcov for suggestions regarding the manuscript. The authors acknowledge financial support from UNIP and UNIFESP. This study was supported by funds from PROSUP, 2013.

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First received 6 October 2016; accepted in revised form 28 November 2016. Available online 6 February 2017