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Brief Report

The Saltiness of The Sea Breaks DNA in Marine Invertebrates

Possible Implications for Animal Evolution

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ABSTRACT

More than 97 percent of the world's water is ocean and its average osmolality of 1000 mosmol/kg is much higher than the 300 mosmol/kg found in most of the intercellular fluids of vertebrates. Many marine invertebrates are osmoconformers, meaning that the osmolality of their extracellular fluid is the same as that of seawater. We report here that marine invertebrates from diverse phyla have numerous DNA breaks in their cells while they are exposed to normal seawater containing high NaCl, but that the DNA breaks decrease or disappear when the animals are acclimated to the same water diluted to 300 mosmol/kg. We speculate that, since DNA breaks cause mutations, salinity might have important background effects on the rate and course of evolution.

Previous studies showed that high NaCl increases the number of DNA breaks in mammalian cells in tissue culture,[1,2] in mouse renal inner medullary cells in vivo[2] and in cells of the soil nematode *Caenorhabditis elegans*.[3] Acute elevation of osmolality from 300 to 500–600 mosmol/kg by adding NaCl to medium bathing mIMCD3 cells increases the number of DNA breaks[1,4] and arrests the cell cycle in all phases.[5,6] The cell cycle arrest is transient. After several hours, the cells adapt and begin proliferating again, despite the continued presence of high NaCl.[7,8] However, even after cells adapt to high NaCl and reenter the cell cycle, numerous DNA breaks persist.[2] Most surprisingly, numerous DNA breaks are also present in normal cells in the mouse renal inner medulla,[2] where NaCl is always high as part of the urinary concentrating mechanism.[9] Elevation of NaCl to an excessive level, which differs between cell types, causes apoptosis.[6] However, it is clear that increased number of DNA breaks that occurs at the more moderate levels of NaCl to which cells can adapt are differ from the chromatin fragmentation that occurs during apoptotic cell death. When high NaCl induces DNA breaks in viable cells, there is no activation of caspases, no nuclear condensation, and no formation of apoptotic bodies, as occurs in apoptosis.[2] Further, high NaCl-induced DNA breaks are reversible, whereas those accompanying apoptotic cell death are not. DNA breaks in cells adapted to high NaCl in tissue culture disappear rapidly when the NaCl is lowered,[2] and those that exist in renal inner medullas of normal mice disappear quickly when the normally high intercellular NaCl concentration is lowered by the diuretic furosemide.[2] The soil nematode, *Caenorhabditis elegans* is able to adapt to and live in a high NaCl environment.[10] Recently we showed that adaptation of *Caenorhabditis elegans* to high NaCl is also accompanied by induction of DNA breaks.[3]

According to some estimates, about 80% of all Earth's life lives in the ocean which has an average osmolality of 1000 mosmol/kg, dominated by NaCl. Findings that high NaCl is accompanied by DNA breaks in both mammalian[2] and *Caenorhabditis elegans*[3] cells suggested the possibility that DNA breaks might be prevalent in all cells bathed by high NaCl, including those of osmoconforming marine invertebrates. Most marine invertebrates, especially soft-bodied ones that have no impermeable coverings to reduce osmotic forces, have NaCl in their extracellular fluids that is as high as in seawater.[11] Therefore, we tested osmoconforming representatives from diverse phyla (Figs. 1 and 2) for DNA breaks. We detected DNA breaks by in vitro labeling of their 3'-OH ends with biotinilated deoxynucleotides in a reaction catalyzed by Terminal Deoxynucleotidyl Transferase (TdT) (TUNEL assay). TdT catalyzes the addition of biotinilated deoxynucleotides to the 3'-OH DNA ends, and the biotin is detected by immunocytochemistry. DNA breaks are present in cells of all these animals while they remain in the seawater from which they were collected (Fig. 3). However, the DNA breaks decrease or disappear in these cells after acclimation of the animals to the seawater gradually diluted to 300 mosmol/kg over a period of five days (which approximates the osmolality of extracellular fluid of humans and other vertebrates)[11]

(Fig. 3). Such decrease of DNA breaks upon lowering the salinity is similar to results seen with mammals[2] and soil nematode.[3]

METHODS

Collection of species. *Procerodis littoralis* (Platyhelminthes, Turbellaria), *Lineus ruber* (Nemertea, Anopla), and *Clitellio arenarius* (Annelida, Oligochaeta) were collected under intertidal rocks at the Marine Science Center, Northeastern University (Nahant, MA). *Nerita peloronta* (Mollusca, Gastropoda) and *Isognomon alatus* (Mollusca, Bivalvia) were collected from intertidal rocks at Knight Key (FL). *Pagurus longicarpus* (Arthropoda, Crustacea) were purchased from Gulf Specimen Marine Labs, Inc (Panacea, FL).

Adaptation to lower osmolality. Animals were either maintained in the same marine water in which they were collected or they were adapted to a lower salinity. The osmolality was lowered to 300 mosmol/kg by diluting the marine water with deionized water gradually over five days.

Detection of DNA breaks. Control animals kept in the original seawater and animals adapted to 300 mosmol/kg were fixed at the same time with ice-cold 4% paraformaldehyde dissolved in PBS at the same osmolality as the water in which they were living. Prior to fixation, animals having a cuticle (*Clitellio arenarius* (Annelida, Oligochaeta) were frozen on dry ice to crack the cuticle and then thawed. *Nerita peloronta* (Mollusca, Gastropoda) and *Isognomon alatus* (Mollusca, Bivalvia) were taken from their shells and cut into small pieces before fixation. *Pagurus longicarpus* (Arthropoda, Crustacea) were fixed inside their shells for 5 minutes, then taken out and put back in 4% paraformaldehyde. All animals were left in 4% paraformaldehyde overnight at 4°C. After fixation, animals were paraffin-embedded, then sections were cut and mounted on silanized slides (American Histolabs, Inc., Gaithersburg, MD). Sections were deparaffinized with xylene and rehydrated in a graded series of dilutions of ethanol. Endogenous peroxidase was quenched by placing the slides in 3% hydrogen peroxide in methanol for 5 min. A DNA fragmentation Detection Kit (#QIA33, Calbiochem, Darmstadt, Germany) was used, according to the manufacturer's instructions, to detect DNA breaks. In brief, the DNA breaks were detected by in vitro labeling of their 3'-OH ends with biotinylated nucleotides, using the reaction catalyzed by Terminal Deoxynucleotidyl Transferase (TdT). TdT catalyzes the addition of biotinylated and unlabeled deoxynucleotides to the 3'-OH ends. Biotinylated deoxynucleotides were detected with streptavidin-peroxidase conjugate. The peroxidase was labeled by addition of 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate, which produces a brown-colored deposit.

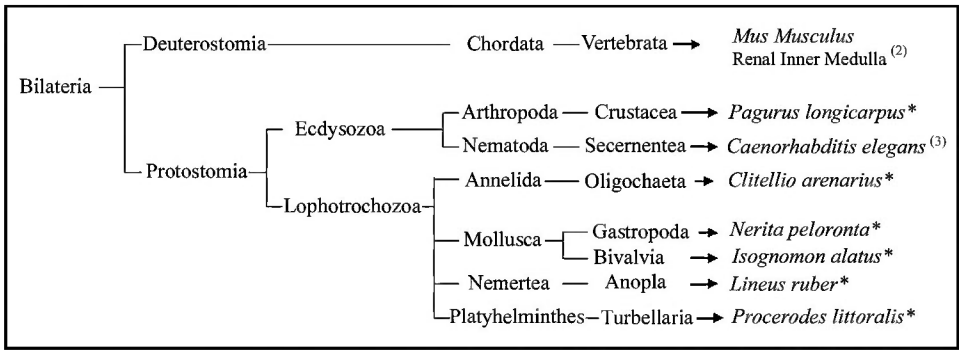

Figure 1. Evolutionary tree of bilaterian animals, showing phyla of those tested for DNA breaks associated with high salinity. (*) present study. (number)previous studies.

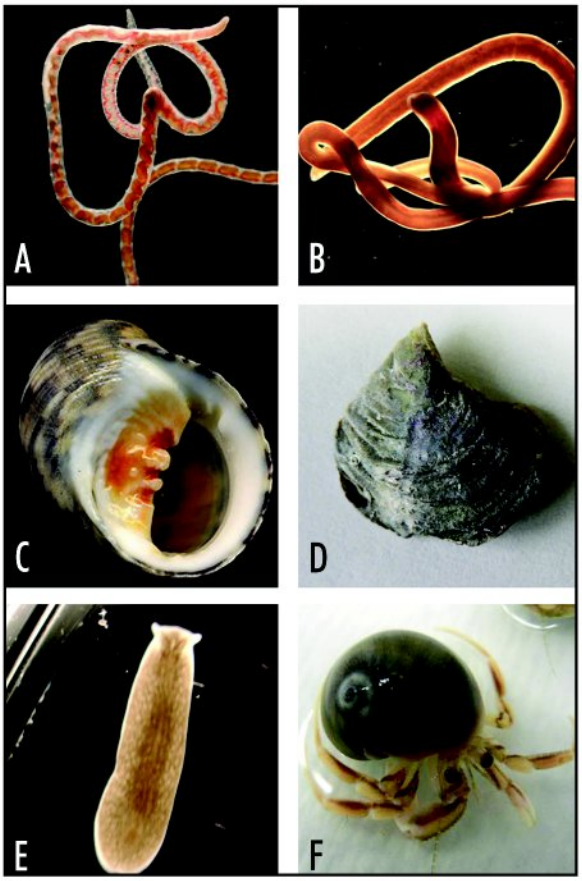

Figure 2. Pictures of marine invertebrates tested for the DNA breaks. (A) *Clitellio orenorius* (Annelida, Oligochaeta); (B) *Lineus ruber* (Nemertea, Anopla); (C) *Nerita peloronto* (Mollusca, Gastropoda); (D) *Isognomon olotus* (Mollusca, Bivalvia); (E) *Procerodis littorolis* (Platyhelminthes, Turbellaria); (F) *Pogurus longicorpus* (Arthropoda, Crustacea).

DISCUSSION

As summarized in Figure 1, high extracellular salinity is accompanied by DNA breaks in all animal phyla tested until now, independent of their complexity and position on the evolutionary tree. These high NaCl-associated DNA breaks could increase the rate of mutation suggesting possible involvement of salinity in creating genetic variability—raw material for natural selection. Indeed, a mutagenic effect of high NaCl has been described in yeast, where exposure of *Saccharomyces cerevisiae* to high NaCl leads to base substitutions and frameshift mutations.[12]

The potential for high salinity to increase mutation rates implicates changes in salinity as possible contributors to changes in evolution rates, for example, to the Cambrian explosive radiation of animal species. Before the Cambrian period began about 544 millions years ago, there was little animal fossil evidence. Then, in the geologically abrupt interval of several million years, an evolutionary explosion littered the fossil record with recognizable remains of every basic

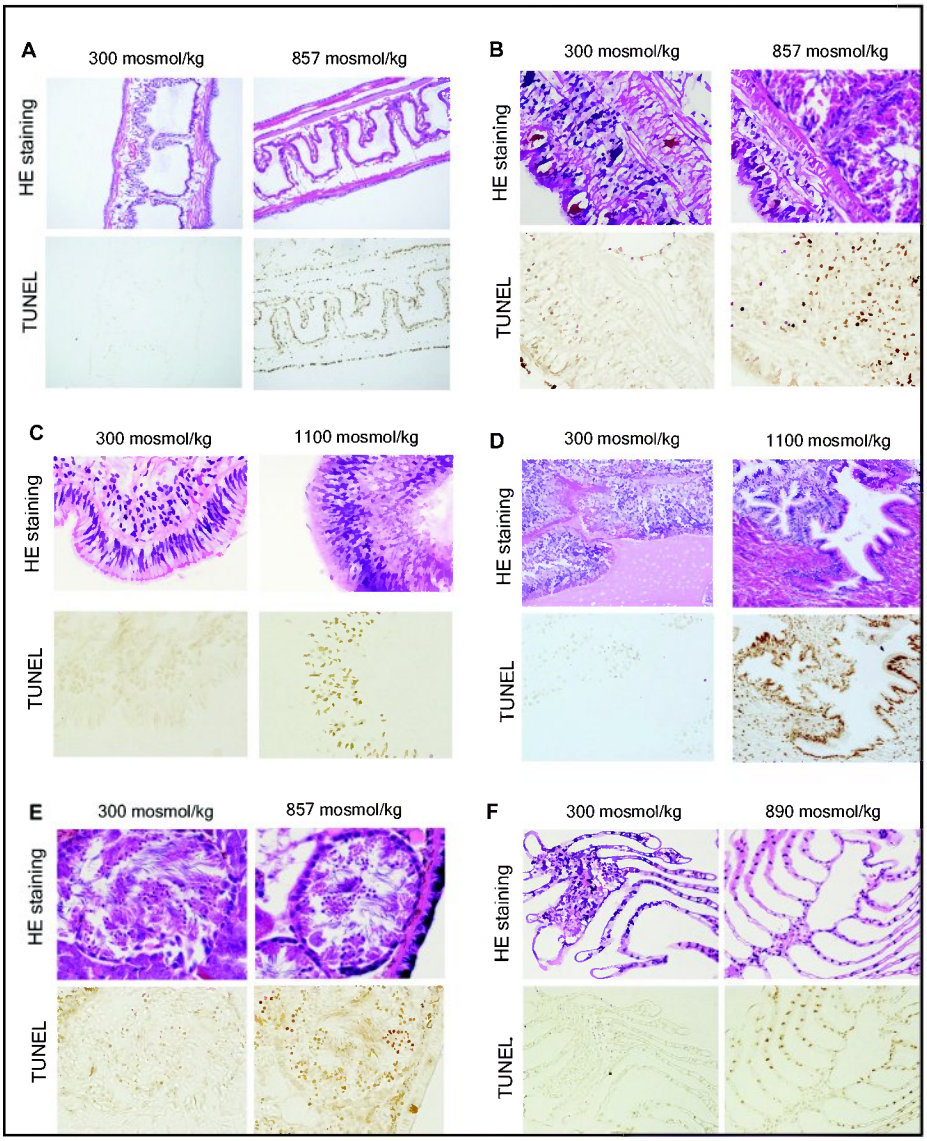

Figure 3. DNA breaks are present in marine invertebrates while exposed to normal marine water but the breaks decrease or disappear after the seawater is diluted to 300 mosmol/kg. Tissue sections were stained with hematoxylin and eosin (HE staining) for general histology: nuclei are blue. DNA breaks were detected on the tissue sections by labeling of 3′-OH ends of broken DNA with biotinylated nucleotides (TUNEL assay): positive labeling is brown. (A) *Clitellio orenorius* (Annelida, Oligochaeta); (B) *Lineus ruber* (Nemertea, Anopla); (C) *Nerito peloronto* (Mollusca, Gastropoda); (D) *Isognomon olotus* (Mollusca, Bivalvia); (E) *Procerodis littorolis* (Platyhelminthes, Turbellaria); (F) *Pogurus longicorpus* (Arthropoda, Crustacea).

form of animal that we know today.[13,14] A great variety of skeletal taxa appeared in rocks estimated to be about 530 million years old,[14] preceded by fossils of soft-bodied animals in rocks about 560 million years old.[15] Cambrian radiation was not restricted to the fauna; at the same time, many algae and protists appeared or radiated.[16] Such coincident events in animals, algae and protozoans suggest a ubiquitous, ecologic trigger. Our results suggest that salinity increase has a potential to be such a trigger. Indeed, conditions were appropriate for local increases in salinity in the Precambrian-Cambrian period. Cambrian diversification coincided with fragmentation of the Late Proterozoic supercontinent Rodinia.[17] Geologic and paleomagnetic data suggest that the supercontinent began to disintegrate 600–500 million years ago.[17] Breakup of megacontinents usually generates an array of epicontinental seas and bays, where water level can fall due to evaporation, leading to increased salinity, as evidenced by formation of thick and often multiple salt layers at the boundaries of continent rifting[18] and at places where such epicontinental seas or bays are located.[19] We suggest that an increased mutation rate, associated with elevated salinity in such basins could have been a factor in explosive radiation. Consistent with this idea, the greatest diversity of fossils is located at the margins of rifting continents in shallow marine sedimentary rocks.[14,15,20-23]

Given that the DNA breaks associated with high extracellular salinity in osmoconforming invertebrates increases the rate of evolution, modern vertebrates with their relatively low and constant extracellular osmolality should have lower incidence of DNA breaks and slower evolution rate. Indeed, estimates from molecular clocks of the rates of evolution show that the rates decreased significantly in vertebrates before the origin of Osteichthyes.[24] It is striking that this slowing coincided with development of precise osmoregulation that maintains interstitial osmolality constant at about 300 mosmol/kg, independent of the salinity of the external environment.[11]

In conclusion, finding of the present study that high salt induces DNA breaks in marine invertebrates together with previous data showing the same phenomenon in mammals[2] and soil nematode[3] suggests that induction of DNA breaks by high extracellular osmolality is a general phenomenon that affects all animals and might affect the rate and course of evolution.

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