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- Note -

Deep Seawater Increases Dendritic Branches of Cultured Rat Hippocampal Neurons

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Deep seawater (DSW; deep ocean water) is pure, rich in inorganic materials which have attracted attention for various applications. In this study we investigated the effects of the DSW upwelled from the East Sea, offshore Yang Yang (Korea) on the morphological differentiation of cultured rat hippocampal neurons, which were grown in the minimal essential medium containing 10% (v/v) fetal bovine serum and 25% (v/v) DSW with various hardness. DSW had no effect on initial morphological differentiation (17 hr post-plating). When observed on DIV3, 7, 14, and 17, low hardness (0 and 200) DSW reduced dendritic branching. However, dendritic branches within 80 μm diameter from the center of soma nearly doubled in neurons grown in hardness 1,000 DSW-containing media. DSW with hardness 600 was more or less same as control groups. These results indicate that DSW with appropriate hardness ameliorates neuronal health.

Key words : Branch, culture, deep seawater, dendrite, hippocampal neuron

Introduction

While no specific definition exists at this time, the deep seawater (DSW; deep ocean water) is generally refers to seawater at depths equal to or greater than 200 meters where Sunlight, which is needed for photosynthesis, does not reach. Wealth of inorganic materials, purity and mineral content have demonstrated DSW's usefulness and attracted attention in various applications. In food and medical fields, DSW has been processed into drinking water, and various beneficial effects have been suggested, but many of them await scientific confirmation.

The DSW was useful for the prevention of hyperlipidemia and arteriosclerosis compared to the surface seawater, and it was found that reduction of the LDL cholesterol level and enhancement of GPx activity were involved in its effects [4]. In a murine model of senile osteoporosis, SAMP6 and its control SAMR1, the DSW increased the stiffness and strength of bone, and the amount of energy absorbed before breaking in femurs from both SAMR1 and SAMP6 [3]. Coordinately, the results from the cell culture study indicated that DSW stimulates both osteoblastogenesis and osteoclastogenesis, i.e., bone turnover by affecting the Ca^{2+} uptake in two types of bone cells [3].

The East Sea is a bowl-shaped 'Ocean Miniature' which is connected to four other open seas through narrow and shallow straits. Due to relatively isolated oceanography, water exchange between the East Sea and other connected oceans are very limited. Therefore, more than 90% of all East Sea water is DSW. In this study we investigated the efficacy of the DSW welled from the East Sea on the health of neurons grown *in vitro*, and show that general morphology is healthier and the number of dendritic branches is larger in DSW-added cultures than distilled water (DW) groups.

Materials and Methods

Deep seawater (DSW)

The DSW was pumped up from a depth of 1,032 m off Yang Yang (Kwang Won-do, Korea) was desalinated and concentrated by reverse osmosis. The desalinated water (hardness 0) and hardness 4,000 DSW were obtained from Waterbis Co., Ltd (AnSan, Korea). The mineral ingredient content of the hardness 4,000 DSW is shown in Table 1 [6].

Neuronal culture

Embryonic day 18 (E19) rat hippocampal cells were dissociated by triturating trypsin-treated tissues, and were

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Table 1. Mineral ingredient content of deep sea water[§]

Mineral ingredient	Desalinated water (mg/l)	Hardness[a)] 4,000 (mg/l)
Ca	0.9	322.0
Mg	2.8	773.2
K	1.0	219.0
Na	4.3	165.1
Cl	17.9	2147.0
SO_4	3.6	1466.0

[a)] hardness: Ca (mg/l)×2.5+Mg (mg/l)×4.1
[§] Cited from Shon et al. (2008)

plated on poly-DL-lysine-coated coverslips (1,000~1,500 cells/mm^2) in the minimal essential medium (MEM; Gibco, #11700-077) containing 10% (v/v) fetal bovine serum (Gibco # 26140-079), 25% (v/v) DSW of various hardness, and 25 μM glutamate, as previously described [1,2]. One third of the culture media was replaced with the fresh one but glutamate every 3 days.

Immunocytochemistry

Cells were fixed through the PFA/MeOH fixation method [5]. Coverslips were rinsed briefly in phosphate buffered saline (PBS) and with 4% paraformaldehyde (PFA) in PBS at room temperature (RT) for 10 min. Coverslips were rinsed in PBS and then incubated in -20°C methanol at -20°C for 20 min. The cells were then rinsed once with PBS and blocked overnight at 4°C in preblocking buffer [5% normal goat serum, 0.05% Triton X-100 in H-PBS (450 mM NaCl and 20 mM phosphate buffer, pH 7.4)]. Primary antibodies [rabbit anti-neurofilament 200 (NF200; 1:250, Sigma) and α-tubulin (1:2,000; mouse monoclonal 12G10, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA) were diluted in preblocking buffer (250 μl per well of 24-well culture plate), replaced with preblocking buffer, and incubated overnight at 4°C. Coverslips were rinsed (15 min x 3) in preblocking buffer, and incubated with secondary antibodies [Alexa Fluor 488-conjugated Streptavidin, Alexa Fluor 568 conjugated-goat anti-rabbit (each diluted 1:1,000 in blocking buffer; Invitrogen)] at RT for 1-2 hr. Coverslips were rinsed once in preblocking buffer for 15 min, twice in PBS, and mounted on slides with 4% n-propylgallate in 90% glycerol and 10% sodium carbonate buffer (pH 8.7).

Fluorescence light microscopy.

A Leica Research Microscope DM IRE2 (Leica Microsystems AG, Wetzlar, Germany) equipped with filter systems I3 S, N2.1 S and Y5 was used to capture light and fluorescent microscopic images. Digital images were acquired with low power (10×) and a HCX PL FL 100X oil-immersion lens and a high-resolution CoolSNAP^{TM} CCD camera (Photometrics Inc., Germany) under the control of a computer equipped with FW4000 (Leica) software. Images (1388×1039 pixels) were processed with the use of Photoshop 5.0 (Adobe Systems).

Analysis

Dendrites within 80 μm diameter from the center of soma of typical pyramidal neurons were counted, and expressed in mean±SD.

Results and Discussion

DSW is clean and rich in mineral components compared to surface seawater [4] and application has been attempted in many fields. DSW has been processed into drinking water, and various beneficial effects have been suggested by many companies. However, scientific evidence is still limited. To our best knowledge, there has been no report at all about the effect of DSW on nervous systems. Therefore, we first set out to investigate the effects of DSW on neuronal health by observing the general morphology of cultured rat hippocampal neurons.

No difference is evident in the early morphological development of cultured neurons.

We dissociated rat hippocampal neurons on embryonic day 18 (E18). At this time point, neurons are round in shape. During the first day after plating onto coverslips *in vitro*, they develop axons and dendrites with growth cones. When the cultures were observed at 17 hr post-plating, neurons in media containing 25% of DSW with various hardness (0-1,000) developed processes with growth cones (Fig. 1. arrows). The speed and number of processes of sample groups did not differ from distilled water (DW)-containing control groups.

Low hardness (0 and 200) DSW is detrimental to dendritic arborization.

The effects of DSW began to appear as early as DIV3. At this stage, about a half of neurons grown in hardness 0 DSW-containing media exhibited very few (often only 2)

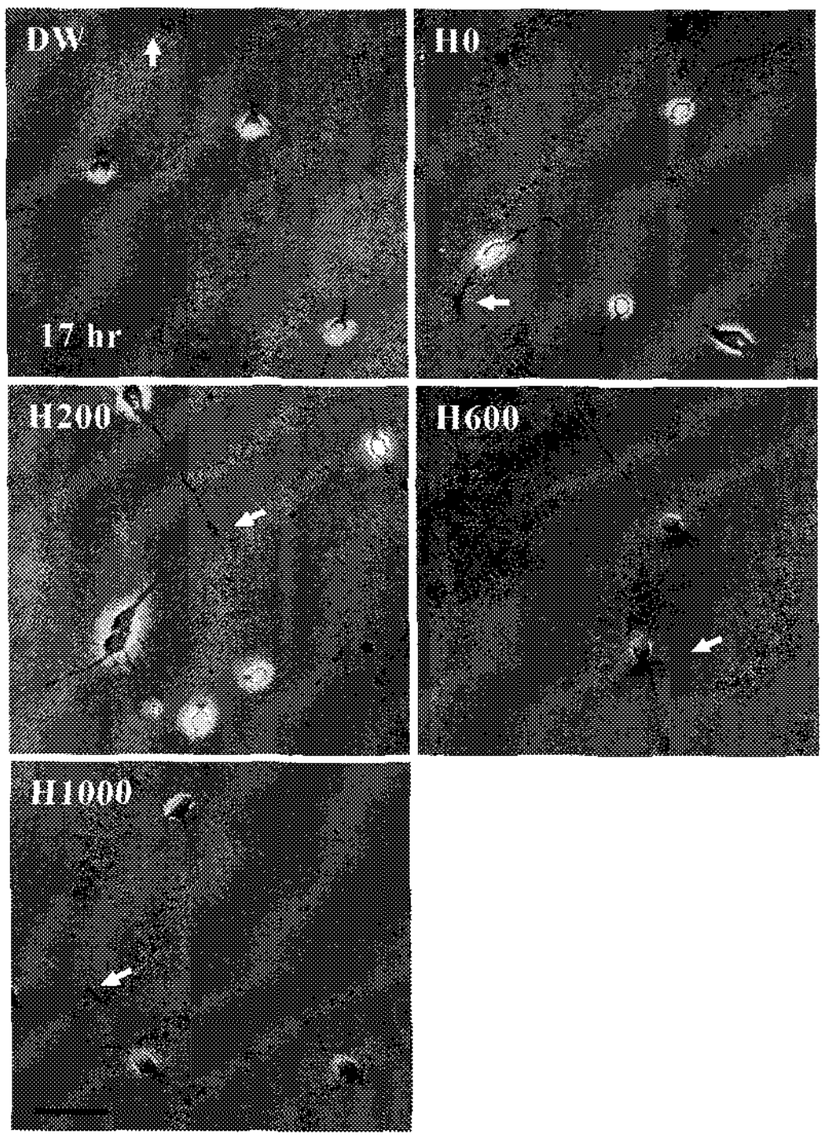

Fig. 1. Light microscopic images of neurons at 17 hr post-plating. Embryonic day 18 (E18) rat hippocampal neurons were dissociated and plated on coverslips in the minimal essential media containing no DSW (DW), hardness 0 (H0), hardness 200 (H200), hardness 600 (H600), and hardness 1,000 (H1000). Growth cones are marked by arrows. Scale bar, 50 μm.

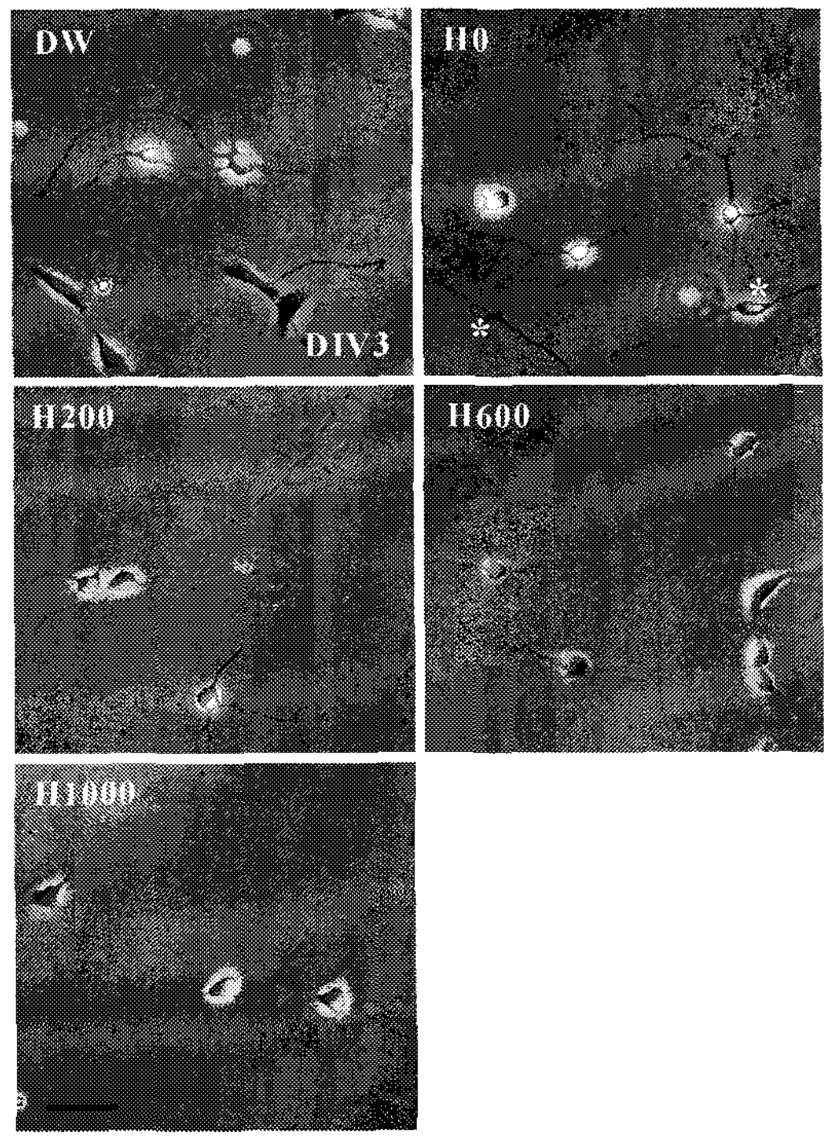

Fig. 2. Light microscopic images of neurons on DIV3. E18 rat hippocampal neurons were grown as in Fig. 1. Neurons with thin bipolar processes are marked with asterisks in H0. Scale bar, 50 μm.

and thin dendrites (Fig. 2, H0, asterisks). Few such neurons were encountered in cultures of control and H200, H600, and H1,000 groups. However, reduced dendritic arborization became evident in the H200, and H0 as well, group on DIV7 (Fig. 3, H200, asterisk). These results indicate that DSW with low hardness has adverse effects on dendritic arborization. Symptoms of unhealthiness were manifested in cultures grown in low hardness (0 and 200) on DIV14. Dendrites of these cultures were often flattened as if they are pressed down by weights (Fig. 4, H0 and H200, arrows), further indicating negative effects of low hardness DSW on neuronal development. Many neurons grown in low hardness (0 and 200) DSW have died by DIV17. The survived neurons exhibited weird morphology. Typical neurons were shown in Fig. 5. Neurons grown in hardness 0 DSW showed very restricted number of dendrites (Fig. 5, H0). Often, they look like bipolar with short, thin, and blunt protrusions (Fig. 5, H0, inset, arrow). Neurons grown in hardness 200 have less number of dendrites than the control group (Fig. 5, H200).

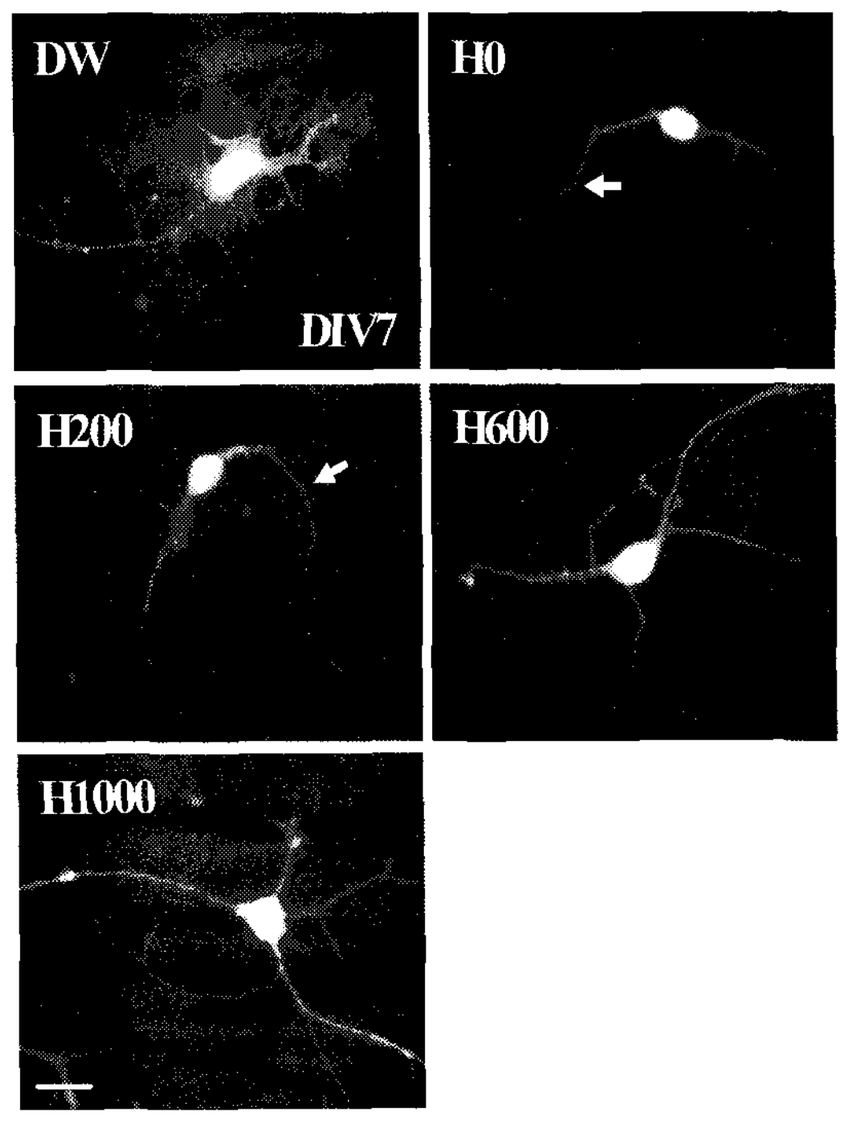

Fig. 3. Fluorescent microscopic images of neurons on DIV7. E18 rat hippocampal neurons were grown as in Fig. 1, and immunostained with an antibody against NF200 to reveal processes. Dendrites with limited branches are marked by arrows (H0 and H200). Scale bar, 20 μm.

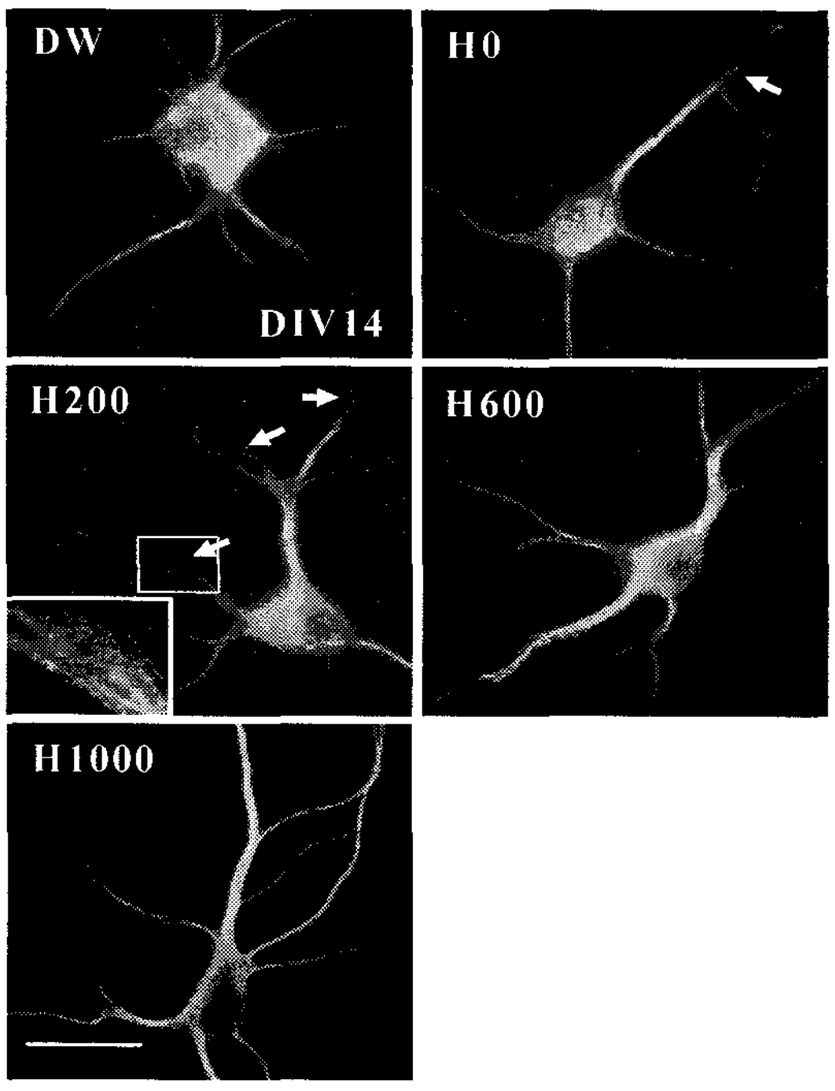

Fig. 4. Fluorescent microscopic images of neurons on DIV14. E18 rat hippocampal neurons were grown as in Fig. 1, and immunostained with an antibody against α-tubulin to reveal processes. Flattened dendrites are marked by arrows (H0 and H200). Scale bar, 20 μm.

DSW with appropriate hardness (1,000) increases dendritic arborization.

Throughout this study we noticed that neurons grown in media containing DSW with hardness 1,000 are healthier than DW-added control groups. These neurons produced dendrites which branch more frequently. Statistic analysis showed that dendritic branches within 80 μm diameter from the center of soma nearly doubled (from 12.5±3.5 to 23.2±3.3). DSW with hardness 600 was more or less same as control groups. This result indicates that DSW with appropriate hardness ameliorates neuronal health and supports neuronal differentiation.

In this study we showed that DSW with low hardness (0 and 200) had adversive effects on dendritic arborization. However, DSW with hardness 1,000 stimulated dendritic branching. The mechanisms for these phenomena are not known. Hardness 0 DSW is the desalinated product of original welled DSW and contains no significant minerals of any kinds (Table 1). The adversive effect of hardness 0 DSW could be due to minor minerals which are not detected. Also, one can not exclude possibilities that the effect is intrinsic to the water itself. For example, the super structure (hydrogen bonding) of water molecules in DSW

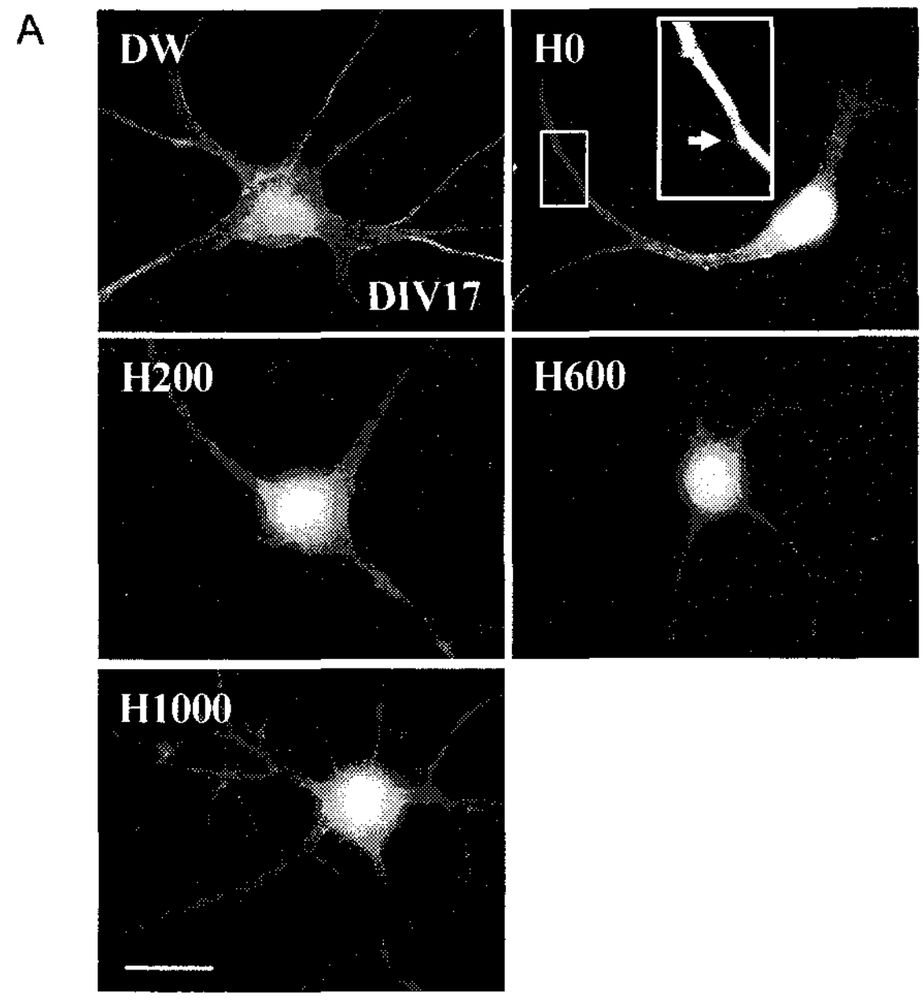

Fig. 5. Fluorescent microscopic images of neurons on DIV17. E18 rat hippocampal neurons were grown as in Fig. 1. (A) Immunocytochemistry. Neurons were immunostained with an antibody against NF200 to reveal processes. Dendrites with limited branches are marked by arrows (H0 and H200). Scale bar, 20 μm. (B) Statistics. Dendritic branches within 80 μm from the center of soma were counted. Double asterisks, $p<0.001$.

may be different from surface water. Also, mysterious is the reason why increase in hardness results in the ameliorating effects on neuronal health. The effect is dose-dependent and reproducible (we repeated 3 times). Increase in hardness may, somehow, have removed minor elements that have adversive effects. Another possibility is that increase in hardness changes the super structure of water.

Efficacy of DSW in other biological phenomena is reported such as in the prevention of hyperlipidemia and arteriosclerosis by reduction of the LDL cholesterol level and enhancement of GPx activity [4], in the increase in stiffness and strength of bone by stimulation of both osteoblastogenesis and osteoclastogenesis [3], and in amelioration of cancer chemopreventive indices [6]. Elucidation of physical

properties of DSW is essential to understand the mechanisms underlying these results including ours. Also necessary are the detailed analyses of the chemical, organic and inorganic compositions of the 'YangYang' DSW, since each DSW may be different site to site.

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References

1. Brewer, G. J., J. R. Torricelli, E. K. Evege and P. J. Price. 1993. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J. Neurosci. Res.* **35**, 567-576.
2. Goslin, K., H. Asmussen and G. Banker. 1998. Rat hippocampal neurons in low-density culture. *In* Banker, G. and K. Goslin. *Culturing nerve cells*, 2nd eds., pp. 339 - 370, Cambridge, MA: MIT Press.
3. Iinuma, Y., K. Motomura, I. Miyagi, F. Maehira and Y. Sudo. 2004. Ameliorative effect of deep-sea water on biochemical and mechanical properties of bone in SAMP6 and SAMR1. *Intl. Congress Series* **1260**, 437 - 440.
4. Miyamura, M., S. Yoshioka, A. Hamada, D. Takuma, J. Yokota, M. Kusunose, S. Kyotani, H. Kawakita, K. Odani, Y. Tsutsui and Y. Nishioka. 2004. Difference between deep seawater and surface seawater in the preventive effect of atherosclerosis. *Biol. Pharm. Bull.* **27**, 1784-1787.
5. Moon, I. S., S. J. Cho, I. Jin and R. A. Walikonis. 2007. A simple method for combined fluorescence in situ hybridization and immunocytochemistry. *Mol. Cells* **24**, 76-82.
6. Shon, Y. H., M. K. Kim, J. S. Jang, E. J. Jung and K. S. Nam. 2008. Effect of deep sea water on phase I, phase II and ornithine decarboxylase. *J. Life Sci.* **18**, 381-386.

초록 : 해양심층수에 의한 해마신경세포 가지돌기 수의 증가

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해양심층수(deep seawater, DSW)는 청정성과 무기물질의 풍부함 때문에 여러 분야에 응용하기 위하여 최근 많은 관심을 받고 있다. 본 연구에서는 동해 양양 부근의 해저 1,100 m에서 취수하여 역삼투압 시스템으로 탈염과 농축을 한 심층수가 배양한 흰쥐해마신경세포의 형태적 분화에 미치는 영향을 조사하였다. 10%(v/v) fetal bovine serum이 첨가된 MEM 배지에서 키운 세포와 비교할 때 25%(v/v) DSW이 포함될 경우 배양 17시간째에는 차이가 없었다. 그러나 DIV3, 7, 14, 및 17에 관찰하면 경도 0 및 200의 DSW가 포함된 배지에서 자란 신경세포는 가지돌기의 수가 현저히 줄었다. 반면에 경도 600의 DSW에서 자란 신경세포는 그 가지돌기의 수가 대조군과 비슷하였으며, 경도 1000의 경우는 대조군에 비하여 거의 2배 증가하였다. 이 결과는 적당한 경도의 DSW는 신경세포의 성장 및 건강을 돕는 것으로 해석된다.