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Original Article

Requirement of protamine for maintaining nuclear condensation of medaka *(Oryzias latipes)* spermatozoa shed into water but not for promoting nuclear condensation during spermatogenesis

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ABSTRACT Protamine is an arginine-rich basic protein found in the sperm nuclei of many vertebrates, but its actual roles in spermatozoa remain to be elucidated. In this study, we investigated the physiological roles of protamine by examining protamine-less spermatozoa produced *in vitro* in the presence of the transcriptional inhibitor actinomycin D. Even under inhibited transcription, medaka spermatocytes underwent meiosis and differentiated into spermatozoa with a condensed nucleus and an elongated flagellum. Using a newly produced anti-medaka protamine antibody, we confirmed the absence of protamine protein in the spermatozoa differentiated in the presence of actinomycin D. These findings clearly indicate that sperm nuclear condensation in medaka is independent of protamine. Since medaka spermatozoa are shed into water upon natural fertilization, we also investigated the roles of protamine by comparing the differences between the nuclear morphology of protamine-equipped and protamine-less spermatozoa immersed in water. The nuclei without protamine more rapidly swelled than did those with protamine and completely broke down within 10 min, whereas more than 80% of the sperm nuclei with protamine resisted the disruption under similar conditions. These findings strongly suggest that a physiological role of protamine in medaka spermatozoa is to protect the ejaculated spermatozoa against the disruption by low osmotic pressure until arrival at the eggs for successful fertilization.

KEY WORDS: *actinomycin D, cell culture, fertilization, medaka sperm, protamine*

Introduction

Spermatozoa are haploid cells that are highly specialized in carrying the male genome to a female germ cell, the egg. They are produced through spermatogenesis, which mainly consists of three processes: mitotic proliferation of spermatogonia, meiosis of spermatocytes, and transformation of spermatids into spermatozoa called spermiogenesis. Spermiogenesis includes condensation of the nucleus, elongation of the flagellum, and discard of the cytoplasm. Protamine, a sperm-nucleus-specific basic protein with a high arginine content, is expressed during spermiogenesis in many vertebrates (Bloch, 1969; Poccia, 1986; Hecht, 1998). Although the biological roles of protamine are still a matter of speculation (Oliva and Dixon, 1991), this protein is thought to be essential for nuclear condensation by its substitution for nuclear histones, since a deficiency in protamine in humans results in, for example, the production of spermatozoa with a large and round head (Balhorn *et al*., 1988; Belokopytova *et al*., 1993). During spermatogenesis of the medaka *Oryzias latipes*, protamine mRNA is expressed in secondary spermatocytes and spermatids and translated into a protein after meiosis (Tamura *et al*., 1994), suggesting the involvement of protamine in nuclear condensation during spermiogenesis also in this species. However, our recent study of spermatogenesis of a hybrid medaka between *O. latipes* and *O. curvinotus* has demonstrated that the spermatozoa of this hybrid contain a condensed nucleus in spite of the absence of protamine mRNA expression (Shimizu *et al*., 1997), a finding raising the possibility that protamine is unnecessary for nuclear condensation during medaka spermatogenesis, in contradiction to the concept that protamine is essential for this process.

Abbreviations used in this paper: AUT-PAGE, acid/urea/Triton X-100 polyacrylamide gel electrophoresis; DIG, digoxigenin; PBS, phosphate-buffered saline.

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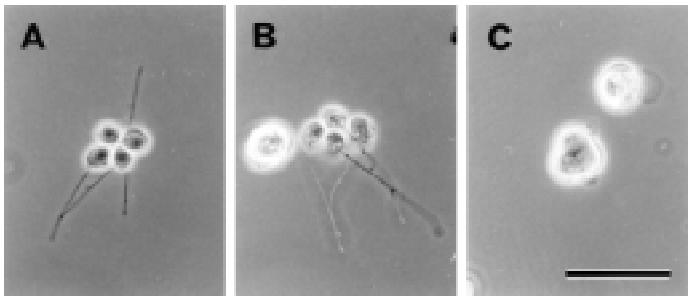

Fig. 1. *In vitro* culture of medaka spermatocytes in the absence (A) or the presence of actinomycin D (B) or cycloheximide (C). *Even under inhibited transcription* **(B)**, *each spermatocyte underwent meiosis and differentiated into four spermatozoa as in the control* **(A)**, *but those treated with the translational inhibitor cycloheximide underwent neither meiosis nor spermiogenesis* **(C)**. *Bar, 12 μm.*

Medaka is a useful experimental animal for investigating regulatory mechanisms of spermatogenesis, because 1) continuous spermatogenesis under artificially controlled environmental conditions allows us to investigate it all the year round, and 2) culture techniques of medaka spermatocytes newly established (Saiki *et al.*, 1997; Shimizu *et al.*, 1997) enable more detailed investigations into control mechanisms of spermatogenesis. In this study, we investigated the physiological roles of protamine, using protamine-less spermatozoa produced *in vitro* in the presence of the transcriptional inhibitor actinomycin D. We clarified the unnecessity of protamine for promoting sperm nuclear condensation by demonstrating its occurrence in the spermatozoa without protamine. Based on the finding that the sperm nuclei lacking protamine are apparently more labile in water than those equipped with protamine, we also suggest that a physiological role of protamine in medaka spermatozoa is to protect their nuclei against disruption in freshwater until reaching the eggs for fertilization.

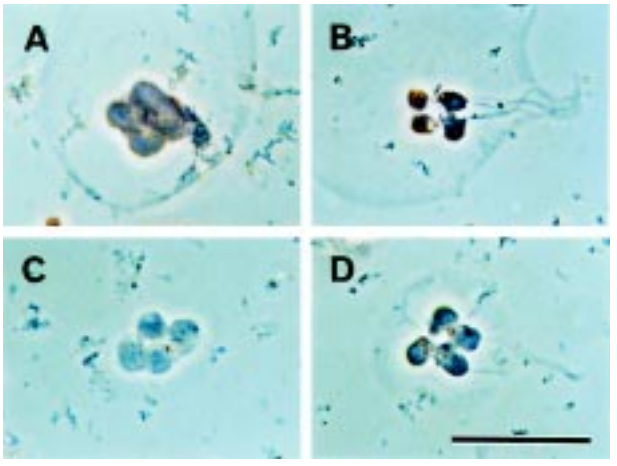

Fig. 2. *In situ* hybridization with DIG-labeled protamine RNA. *Spermatids* **(A,C)** *and spermatozoa* **(B,D)** *formed in vitro in the absence* **(A,B)** *or the presence of actinomycin D* **(C,D)** *were hybridized with the antisense probe. DIG signals appear in brown. Note the presence* **(A,B)** *and the absence* **(C,D)** *of protamine mRNA expression in actinomycin D-untreated and -treated spermatogenic cells, respectively. The differences between the signal intensities of right and left two spermatozoa shown in* **(B)** *are an artifact due to the differences in focal plane; the cytoplasm is in focus in the left while the nucleus is in focus in the right. Dot-like signals found in the cytoplasm of spermatogenic cells in* **(C,D)** *are detectable when the sense probe is used, thereby being non-specific. Bar, 20 μm.*

Results

Production of spermatozoa in vitro

Under culture conditions, a primary medaka spermatocyte was able to differentiate into four spermatozoa each having a condensed nucleus and an elongated flagellum after undergoing meiosis and spermiogenesis (Fig. 1A), as described previously (Saiki *et al.*, 1997; Shimizu *et al.*, 1997). *In situ* hybridization analyses with an antisense riboprobe showed that the spermatids and spermatozoa produced *in vitro* expressed protamine mRNA (Fig. 2A,B), as is the case *in vivo* (Tamura *et al.,* 1994). To examine protamine protein in the spermatogenic cells, we produced an antibody against a synthetic peptide corresponding to the C-terminal half of medaka protamine (see Materials and Methods).

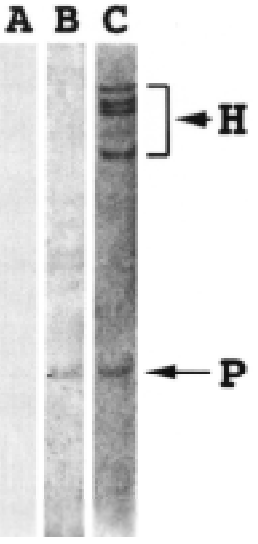

Fig. 3. Anti-protamine immunoblotting of the basic nuclear proteins extracted from medaka spermatozoa. *The proteins separated by AUT-PAGE were stained with preimmune serum* **(A)**, *anti-protamine antibody* **(B)** *and Coomassie brilliant blue* **(C)**. *The antibody recognizes protamine (P) but not histones (H).*

The specific recognition of medaka protamine by this antibody was confirmed by immunoblotting sperm nuclear extracts separated by acid/urea/Triton X-100 polyacrylamide gel electrophoresis (AUT-PAGE) (Fig. 3). Immunocytochemistry with the newly produced anti-medaka protamine antibody confirmed the presence of protamine protein in the *in vitro*-produced sperm nucleus (Fig. 4A,B). In addition, we previously demonstrated that the *in vitro*-produced spermatozoa could fertilize the eggs and produce normal embryos (Saiki *et al.,* 1997). Thus, it is concluded that the spermatozoa produced *in vitro* have similar biological characteristics to those produced *in vivo.*

Production of spermatozoa without protamine

To produce spermatozoa without protamine, we cultured primary spermatocytes in the presence of the transcriptional inhibitor actinomycin D. Even in the presence of actinomycin D, the spermatocytes underwent both meiosis and spermiogenesis and differentiated into spermatozoa with an elongated flagellum and a condensed nucleus (Fig. 1B). Electron microscopic observations indicated that the nuclei

of spermatozoa produced in the presence of actinomycin D were condensed to the same extent as those of spermatozoa without inhibitors (Fig. 5A,B). For confirming the inhibition of protamine expression by actinomycin D, the treated cells were examined by *in situ* hybridization with the antisense protamine RNA probe and by immunocytochemistry with the anti-protamine antibody. The actinomycin D treatment completely inhibited the expression of protamine mRNA (Fig. 2C,D) and protein (Fig. 4C,D). In contrast to actinomycin D treatment, spermatocytes underwent neither meiosis nor spermiogenesis in the presence of the translational inhibitor cycloheximide (Fig. 1C). These results clearly indicate the occurrence of nuclear condensation without protamine during medaka spermatogenesis. These results also indicate that the progression of meiosis and spermiogenesis are mainly under the translational control of mRNAs that have already been stored in primary spermatocytes, which we discussed in a recent paper (Mita *et al.,* 2000).

Resistance of sperm nucleus to water

In the wild, spermatozoa are ejaculated into water for fertilizing eggs. To investigate the biological roles of protamine, we therefore examined the differences between the resistance to water of spermatozoa equipped with protamine and those without protamine. The protamine-equipped sperm nuclei were considerably resistant to water (Figs. 6A,7A). About half (41.7%) of the nuclei remained intact for 2 min in water. During a 10-min observation period, 15.8% of the nuclei remained unchanged, 76.3% swelled, and 7.9% broke down (Fig. 6A). On the contrary, the protamine-less sperm nuclei were highly labile in water (Figs. 6B,7B). The majority of the nuclei (94.1%) rapidly swelled within 1 min and all of them broke down within 10 min (Fig. 6B).

Discussion

Protamine is an arginine-rich basic nuclear protein found in the spermatozoa of many animal species. Generally, sperm nuclei condense tightly during spermiogenesis. Because nuclear condensation usually coincides with the replacement of preexisting histone with newly produced protamine, protamine is believed to play a key role in this process. In fact, it has been reported that a deficiency in human protamine 2 yields infertile spermatozoa with large and round heads (Balhorn *et al.*, 1988; Belokopytova *et al.*, 1993). This notion is also supported by the finding that goldfish sperm nuclei, which have no protamine, are not fully condensed as compared with those of other fishes having protamine (Muñoz-Guerra *et al.,* 1982). However, our previous finding that sperm nuclei of an interspecific hybrid between *Oryzias latipes* and *O. curvinotus* undergo nuclear condensation without protamine mRNA expression (Shimizu *et al.*, 1997) raised the possibility that protamine is not primarily involved in initiating sperm nuclear condensation in medaka. Here, we provide for the first time strong evidence that medaka sperm nuclear condensation is independent of protamine by having demonstrated that sperm nuclei of wild type medaka, as well as the hybrid (Shimizu *et al.,* 1997), are condensed even in the absence of protamine.

It is concluded that protamine is not involved in nuclear condensation during medaka spermatogenesis. We have also shown that nuclear condensation of medaka spermatozoa occurs under continuous inhibition of transcription starting from primary spermatocytes, indicating that the mRNAs for proteins responsible for nuclear condensation must have already existed in primary spermatocytes. In *Xenopus*, sperm nuclei are thought to be condensed by the aid of sperm-specific basic proteins different from protamine, a major component of which (SP4) is transcribed in primary spermatocytes (Hiyoshi *et al.,* 1991; Mita *et al.,* 1991; Yokota *et al.,*

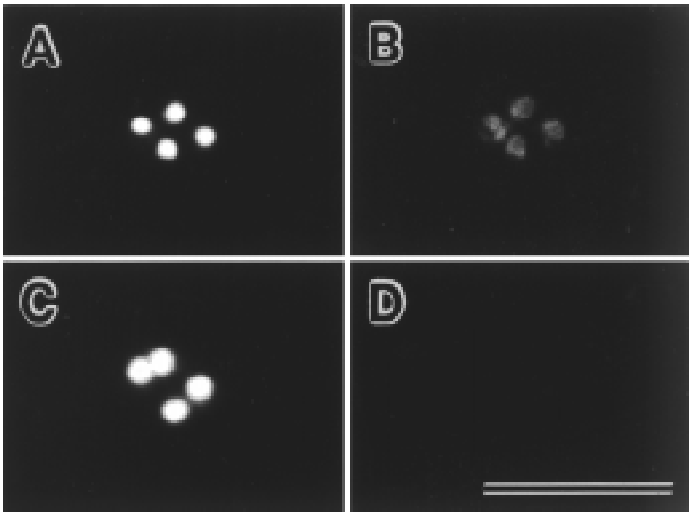

Fig. 4. Anti-protamine immunocytochemistry of spermatozoa differentiated *in vitro* in the absence (A,B) or the presence of actinomycin D (C,D). *Spermatozoa were simultaneously stained with the DNA staining dye Hoechst 33258* **(A,C)** *and anti-protamine antibody* **(B,D)**. *The spermatozoa produced under inhibited transcription lack protamine proteins. Bar, 20 μm.*

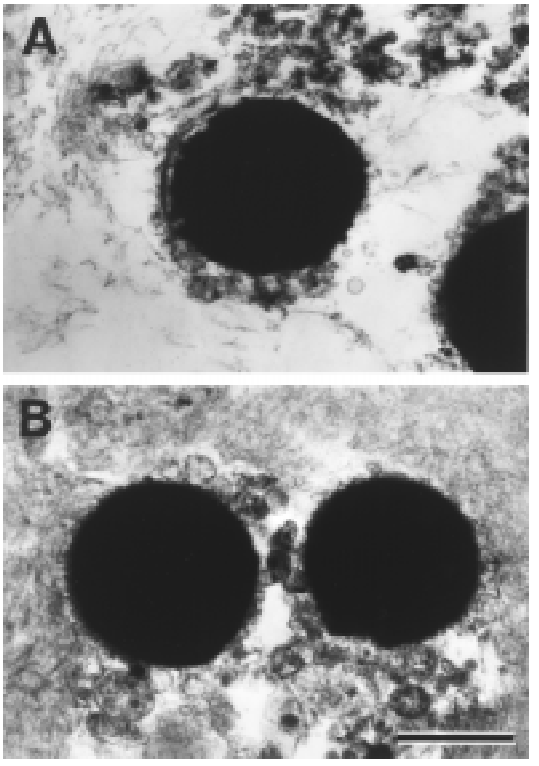

Fig. 5. Electron micrographs of sperm nuclei differentiated without (A) or with actinomycin D (B). *Sperm nuclei condensed even under inhibited transcription* **(B)**. *Bar, 1 μm.*

1991). This situation is consistent with that in medaka shown in this study. Like in *Xenopus*, it is plausible that sperm nuclear condensation in medaka is promoted by sperm-specific basic proteins, but not by protamine, the transcriptions of which are initiated at the latest in primary spermatocytes, although we must await biochemical characterization of these proteins responsible for medaka sperm nuclear condensation.

We have revealed that protamine does not function in promoting nuclear condensation during medaka spermatogenesis. Then, what are protamine's roles in this species? Bloch (1969) proposed that, besides its role in nuclear condensation necessary to inhibit gene activity, protamine might be involved in the protection of condensed nuclei against adverse environmental conditions until the spermatozoa reach the eggs. In many freshwater fishes, in which spermatozoa are ejaculated into water for fertilizing eggs, spermatozoa must resist water invasion into the sperm cells by low osmotic pressure until arrival at the eggs. In this study, we demonstrated that, when treated with water, most of the nuclei that contain no protamine swelled rapidly within 2 min and all of them were disrupted within 10 min, in striking contrast to the nuclei equipped with protamine, almost all of which (ca. 92%) resisted water invasion for at least for 10 min. The swelling of sperm nuclei in water within a few minutes must cause a severe defect in fertilizability, because medaka spermatozoa must pass through the narrow micropyle before entering the egg cytoplasm (the diameter of the inner opening of micropyle being 3.5 μm and the diameter of sperm head being 1.7 μm, Iwamatsu *et al.,* 1997). It has been reported in medaka that the time required for the spermatozoa to reach the eggs following insemination depends on the concentration of spermatozoa, varying from 5 to 25 sec at

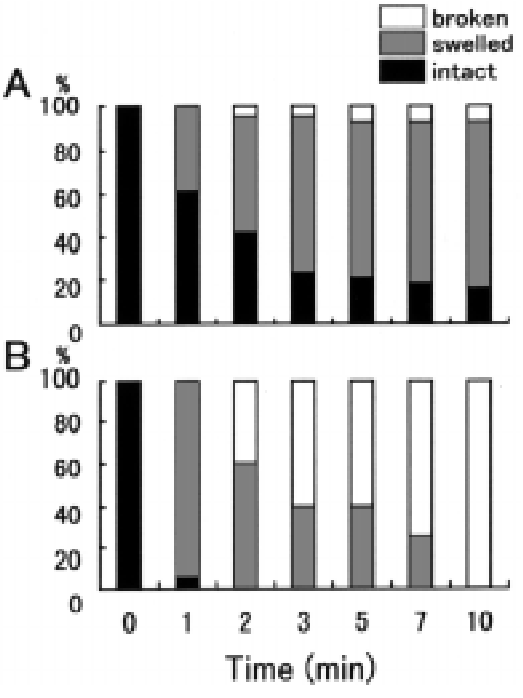

Fig. 6. Changes in morphology of sperm nuclei immersed in water. *Spermatozoa produced in vitro in the absence* **(A)** *or the presence* **(B)** *of actinomycin D were immersed in water and recorded with a video camera for 10 min. Changes in sperm nuclear morphology are shown by the percentage of the sperm nuclei classified into three categories (intact, swelled and broken).*

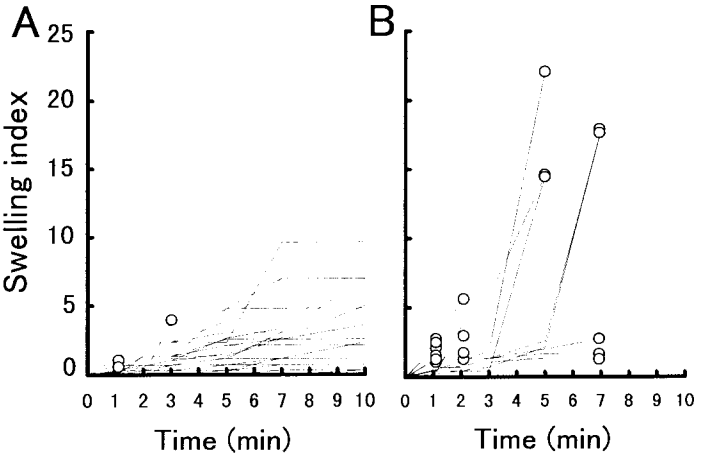

Fig. 7. Swelling of sperm nuclei in water. *From the same data used for Figure 5, the swelling index of the spermatozoa without* **(A)** *or with actinomycin D* **(B)** *was calculated (see Materials and Methods). Open circles indicate the disruption of sperm nuclei at the indicated times during the 10-min observation period.*

concentrations of $20x10^7$ to $1x10^7$/ml (Iwamatsu *et al.*, 1991). It is reasonable to assume that at a relatively low concentration of the spermatozoa, which might frequently occur under natural fertilization conditions, the time required for successful fertilization is much longer, in the order of minutes. Thus, it is highly likely that the spermatozoa unequipped with protamine are unable to participate in fertilization under natural conditions, because of their failure to pass through the narrow micropyle due to their rapid swelling and disruption in water.

Taken together, the findings strongly suggest that medaka protamine plays an essential role in successful fertilization by protecting the spermatozoa from disruption and swelling during the process from ejaculation into water until entering the egg cytoplasm via the micropyle. In this experiment, however, we cannot exclude the possibility that actinomycin D inhibits the expression of proteins other than protamine necessary for nuclear protection. Further investigations in which protamine expression and function are specifically inhibited by injecting the antisense RNA and antibody, respectively, are necessary for verifying the biological role of protamine proposed in this study.

Materials and Methods

Medaka and cell culture

An orange-red variety of medaka was purchased from a local fish farm in Aichi Prefecture and cultured under reproductive conditions (14-h light and 10-h dark at 28°C). Spermatocytes were isolated from testes and cultured on poly-L-lysine-coated cover slips as described previously (Shimizu *et al.*, 1997). Transcription and translation were inhibited by 2 μg/ml actinomycin D and cycloheximide, respectively.

Electron microscopic observation

Spermatozoa differentiated *in vitro* were observed by electron microscopy as described previously (Shibata and Hamaguchi, 1986, 1988). Briefly, spermatozoa on cover slips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS: 102.4 mM NaCl, 1.6 mM KCl, 6.4 mM NaH_2PO_4, 1.6 mM KH_2PO_4, pH 7.4), dehydrated with ethanol, embedded in epoxy resin, and observed under an electron microscope.

In situ hybridization

Expression of protamine mRNA was examined by *in situ* hybridization using digoxigenin (DIG)-UTP-labeled probes transcribed *in vitro* from a medaka protamine cDNA (Tamura *et al.,* 1994). Spermatozoa were washed with PBS and fixed with 4% paraformaldehyde in PBS for 4 h at 4°C. Preparation of the DIG-labeled probe, conditions of hybridization and washing, and detection of the signal were performed according to manufacturer's instructions (Boehringer, Tokyo, Japan).

Immunological detection of medaka protamine

The anti-medaka protamine polyclonal antibody (antiserum) was raised against a synthetic peptide, CVRRTRVVRRRRRVGR, which corresponds to the C-terminal half of medaka protamine (named MP-1, Tamura *et al*., 1994) with an additional cysteine in the N-terminus. The peptide was conjugated to bovine serum albumin as described previously (Kajiura *et al*., 1993) and injected into two mice. Antisera obtained from the two mice exhibited similar titer and specificity. Thus, we used one of the antisera throughout this study.

Basic nuclear proteins were extracted from medaka spermatozoa according to Mann *et al*. (1982). The proteins were separated by AUT-PAGE (Zweidler, 1978) and immunoblotted as described previously (Yamashita *et al*., 1991).

Immunocytochemistry was performed as follows. Spermatozoa were fixed with 4% paraformaldehyde in PBS for 4 h at 4°C. Mouse anti-protamine serum (1:10 dilution, this study) and Cy2-conjugated anti-mouse Ig (1:1000 dilution, Amersham Pharmacia Biotech, Tokyo, Japan) were used as a primary and a secondary antibody, respectively. Specimens were mounted with 10 μg/ml Hoechst 33258 (Calbiochem, La Jolla, CA) and observed under a fluorescence microscope.

Morphological changes in the sperm nucleus in water

Spermatozoa were washed with PBS and treated with distilled water under a light microscope. Changes in sperm nuclear morphology were recorded with a video camera (CTV-270, Shimadzu, Kyoto, Japan) and shown by the percentage of the sperm nuclei classified into three categories (intact, swelled and broken) during a 10-min observation period. Using the data recorded, sperm nuclear volume was also calculated from its diameter and expressed as the swelling index (V_t-V_0/V_0, where V_t indicates the sperm nuclear volume observed at time t and V_0 indicates the initial volume at time 0). Similar experiments using tap water instead of distilled water gave essentially the same results.

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