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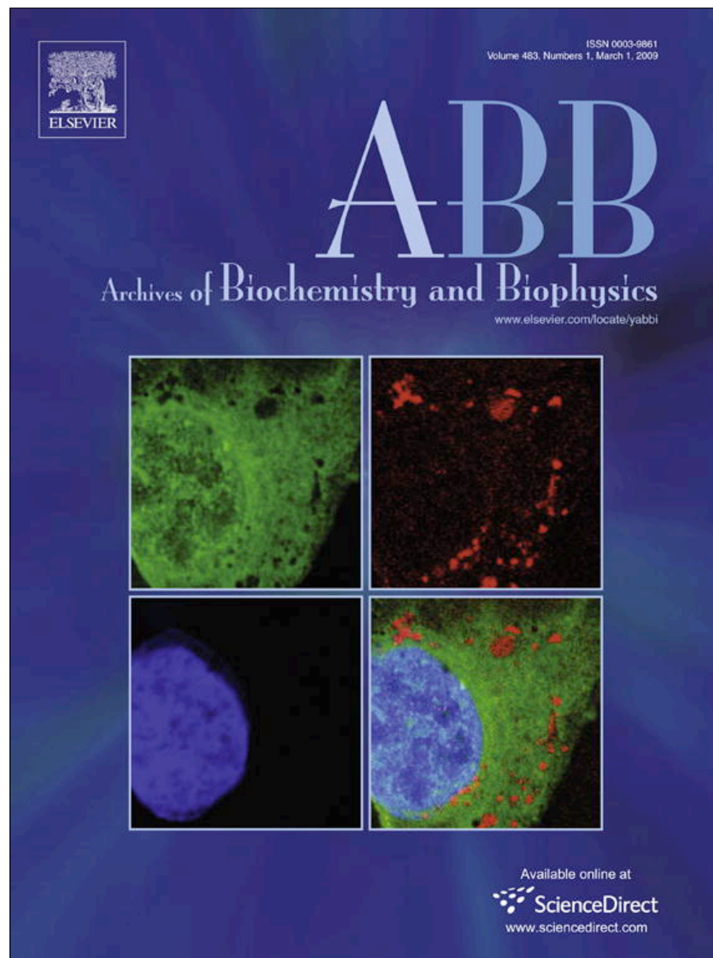

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The sea urchin embryo: A model to study Alzheimer's beta amyloid induced toxicity

M. Pellicanò [a], P. Picone [a,b], V. Cavalieri [c], R. Carrotta [d], G. Spinelli [c], M. Di Carlo [a,*]

[a] Istituto di Biomedicina ed Immunologia Molecolare, CNR, via Ugo La Malfa 153, 90146 Palermo, Italy
[b] Dipartimento di Chimica e Tecnologie Farmaceutiche, Università di Palermo, via Archirafi 32, 90123 Palermo, Italy
[c] Dipartimento di Biologia Cellulare e dello Sviluppo, Università di Palermo, Parco d'Orleans II, Palermo, Italy
[d] Istituto di Biofisica, CNR, via Ugo La Malfa 153, 90146 Palermo, Italy

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ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia. The cause of AD is closely related to the accumulation of amyloid beta peptide in the neuritic plaques. The use of animal model systems represents a good strategy to elucidate the molecular mechanism behind the development of this pathology. Here we use the *Paracentrotus lividus* embryo to identify molecules and pathways that can be involved in the degenerative process. As a first step, we identified the presence of an antigen related to the human APP, called *Pl*APP. This antigen, after gastrula stage, is processed producing a polypeptide of about 10 kDa. By immunohistochemistry we localized the *Pl*APP antigen in some serotonin expressing cells. Similarly, after 48 or 96 h incubation, a recombinant β-amyloid peptide, rAβ42, accumulates around the intestinal tube and oesophagus. In addition, incubation of sea urchin embryos with two different solutions rich in oligomers and fibrillar aggregates of rAβ42 induce activation of apoptosis as detected by TUNEL assay. Moreover, we demonstrate that aggregates induce apoptosis by extrinsic pathway activation, whereas oligomers induce apoptosis both by extrinsic and intrinsic pathway activation. Utilizing an apoptotic inhibitor, caspases activation was offset and morphological damage rescued. Taken together all these observations suggest that the sea urchin may be a simple and suitable model to characterize the mechanism underlining the cytotoxicity of Aβ42.

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Introduction

Alzheimer's disease is a common pathological process affecting the elderly, causing progressive loss of cognition. The morphologic evidences are both extracellular amyloid proteinaceous deposits, called amyloid plaques and intracellular neurofibrillary tangles. The major constituent of the amyloid plaques is a hydrophobic peptide, called amyloid beta protein (Aβ). Aβ is a 39–42 amino acid peptide deriving from multiple proteolytic cleavage at the C-terminal position of a large transmembrane protein, the amyloid precursor protein (APP)[1] [1–4]. Aβ is able to self-assembly and form ordered fibril aggregates, typically rich of β-sheet structures that result to be a key point of the neurodegeneration process. Aβ, indeed, can form oligomers, fibrils and larger clumps of aggregates, both *in vivo* and *in vitro*, depending on the environment conditions [5,6]. More recent studies have suggested that just these Aβ intermediates and in particular Aβ-derived diffusible ligands (ADDLs) are important in AD pathogenesis stimulating the inflammation process and neurodegeneration [7,8]. Whether the aggregation of the peptide into amyloid deposits constitutes clearance of a toxic soluble entity or the amyloid plaques themselves are toxic, is still debated. Several reports sustain the hypothesis that formation of soluble oligomers could play a role at the onset of the pathology [9,10]. The involvement at this stage of the oligomeric species as real cause of the disease has suggested interesting strategies to achieve premature diagnosis of the pathology [11,12].

However, the molecular mechanisms that trigger neurodegeneration are not yet well known. Different studies have identified the apoptotic process as the path for Aβ cell death. However, also in this case, the possibility of a correlation between Aβ oligomeric or aggregates forms and specific apoptotic pathways remains unclear.

APP is a protein conserved during evolution and APP-related genes has been found also in less evolutes organisms such as *Drosophila melanogaster* and the worm *Caenorhabditis elegans*, suggesting that common mechanisms and answer to pathological stimuli could be conserved [13–15]. To utilize a simple model system able to mimic at least a few original mechanisms of a disease, open new possibilities to test therapeutic strategies. Recently, we have employed the sea urchin to investigate about a different toxicity between Aβ oligomers and aggregates [16]. In this kind of experiment sea urchin embryos were separately incubated with a

* Corresponding author. Fax: +39 0916809548.
E-mail address: di-carlo@ibim.cnr.it (M. Di Carlo).

[1] *Abbreviations used:* AD, Alzheimer's disease; APP, amyloid precursor protein; ADDLs, Aβ-derived diffusible ligands; ACh, acetylcholine; ThT, thioflavin-T; ABAD, Aβ-binding alcohol dehydrogenase.

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recombinant Aβ42 (rAβ42) peptide dissolved under different conditions at pH 7 or pH 3, to obtain small oligomers or large aggregates, respectively [16]. By the percentage of the survived or morphologically altered embryos it was possible to demonstrate that small oligomers are more toxic than large aggregates. In line with this study, another sea urchin species, *Sphaerechinus granularis* was utilized to establish a potential protective role played by acetylcholine (ACh), and other neurotransmitters such as serotonin (5HT) and cannabinoids by Aβ42 produced damage [17]. With the idea of utilizing the *Paracentrotus lividus* sea urchin as a model system to study Aβ induced toxicity, we have identified a *Pl*APP antigen localized in same cells corresponding to the sea urchin primordial nervous system. Furthermore, we report evidence about activation of cell death pathway both by Aβ oligomers and aggregates, suggesting that the mechanism of the sea urchin cellular answer to Aβ treatment can be compared to the human one. In turn, addition of anti-apoptotic compounds reverse caspase activation, sustaining the idea that sea urchin could be employed for future therapeutic strategies.

Materials and methods

Protein extraction and Western blotting

Total proteins were prepared by pulsed sonication for 90 s. in Solubilising buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 2 mM PMSF, 10 μg/ml protease inhibitor, 1 mM Na_3VO_4, 1 mM NaF) from embryos at different development stages in ice. Protein samples (50 μg) were electrophoretically separated using 12% SDS–PAGE gel and transferred onto nitrocellulose filters for immunoblotting. After blocking in 3% BSA in PBST (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2PO_4, pH 7.4, 2% Triton X-100), the Western blot was incubated with human anti-Aβ (1:200; Santa Cruz). Primary antibody was detected using the ECL chemiluminescence kit (Amersham) according to the manufacturer's instructions, and using an anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:5000) (Amersham).

Immunohistochemistry of endogenous and exogenous APP

Paracentrotus lividus embryos were cultured in sea water (SW) without or with 1 μM of rAβ42 oligomers and aggregates [16] for 48 and 120 h. After washing in SW were fixed in freshly prepared 4% paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2PO_4, pH 7.4) for 30 min, and kept at 4 °C. After three washes in PBS, the slides were incubated with for 1 h with 3% bovine serum albumine/PBS. Then the embryos were incubated with human anti-Aβ (1:200; Santa Cruz), or with anti-serotonin (1:100; SIGMA) or anti-His (Penta His Alexa fluor conjugate) (1:100; QIAGEN) at 4 °C overnight. After three washes in PBST (PBS, 2% Triton X-100) the samples treated with anti-Aβ were incubated with anti rabbit TRITC-conjugate secondary antibody (1:300), and the samples treated with anti-serotonin were incubated with anti-rabbit FITC-conjugate secondary antibodies (1:300), whereas the samples treated with anti-His were directly detected. Fluorescent or light field images were observed with an Axioscop 2 microscope (Zeiss) and captured with an Axiocam digital camera (Zeiss) interfaced with a computer.

Preparation and characterization of Aβ42 oligomers and fibrillar aggregates

Isolation and purification of the rAβ42 and the preliminary treatment with TFA have been reported elsewhere [16]. To obtain small oligomers, the powder of rAβ42 was dissolved in 0.01 M Tris–HCl buffer pH 7.2, while to obtain large fibrillar aggregates, a solution of rAβ42 was dissolved in 0.1 M Na–citrate buffer pH 3.1 and hold at $T = 37$ °C for 4 days. In the former case the solution was readily characterized by dynamic light scattering at $T = 15$ °C and then frozen in aliquots to be used for different experiments. In the latter case the solution at acid pH was also characterized by dynamic light scattering at $T = 15$ °C after 4 days incubation at $T = 37$ °C and then frozen in aliquots to be used for different experiments.

Thioflavin T staining

Recombinant Aβ42 was dissolved at pH 3.1 and pH 7.2 at a concentration of 340 μM and incubated at 37 °C. Aliquots immediately after dissolution (time 0), and after 48 and 96 h of incubation were taken and stained adding thioflavin-T at a final concentration of 70 μM and applied to microscope slides. The presence of large fibrillar aggregates with dimensions in the range of micrometers was visualized with fluorescence optics of an Axioscop 2 microscope (Zeiss, USA). The images were captured using an Axiocam digital camera interfaced with a computer.

Paracentrotus lividus morphogenetic assays

Eggs from sea urchins (*Paracentrotus lividus*) were demembranated by fertilization in 2 mM PABA. Approximately 1000 embryos were added to artificial sea water (ASW) and purified rAβ42 was added in 8-well plates at two cell stages and the embryos were left to develop. Depending on the experiment, the rAβ42 was solved in water (to obtain a mixture of oligomers and fibrillar aggregates, ranging between 5 and 100 nm) or in buffer at pH 3.1 or pH 7.2 and utilized at different doses (0.6 or 1.5 μM). Control embryos, without any treatment or with specific buffer, were also cultured. The effect on morphogenesis was observed at different times of development by microscopic inspection using a Axioscop 2 microscope (Zeiss, USA). The effect on morphogenesis was monitored before to utilize the embryos for any successive assay. For z-VAD-fmk experiments 30 μM of z-VAD-fmk (PROMEGA) was added together 0.6 μM of rAβ42 oligomers before first cleavage and the embryos were cultured for 48 h. As control untreated or 0.6 μM rAβ42 treated embryos were utilized. All the images were captured using an Axiocam digital camera interfaced with a computer.

Apoptosis assays

TUNEL assay was performed according to the manufacturer instructions (PROMEGA). Briefly, embryos after treatment with 1.5 μM of rAβ42, both under oligomers or aggregates forms, for 24 h were fixed with 4% paraformaldehyde in PBS for 30 min., washed with PBS permeabilized with 0.2% Triton X-100 in PBS for 5 min, rinsed with PBS, and incubated with TUNEL reaction mixture (enzyme, nucleotides) in an humidified atmosphere at 37 °C for 1 h. Staining was obtained by using a peroxidase substrate, hydrogen peroxide, and the stable chromogen, diaminobenzidine (DAB). Following these incubations, samples were rinsed three times with PBS and analysed under Zeiss Axioscop 2 microscope.

Caspase assays

Caspase 8, 9, 3 activities in embryos were measured using commercially available luminescent assays (caspase-Glo™ −8, −9, −3/7 assay systems, PROMEGA). *P. lividus* embryos at gastrula stage were treated with 1.5 μM oligomers or aggregates for 4 h. Untreated embryos were utilized as control. Caspase reagent specific for each kit was added directly to the cells in white 96-well plates and after mixing, were incubate for 15–30 min before recording luminescence with ^{wallac}Victor^2 1420 MULTILABEL COUNTER (Perkin-Elmer) apparatus. The caspase activator assay was performed utilizing 20 μM vinblastine (SIGMA) and the caspase inhibitor assay was performed utilizing 50 μM z-VAD-fmk (PROMEGA). Caspase −8, −9, −3 activity are expressed as a relative percentage of

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untreated samples as 100%. ASW was utilized as blank control. The experiment was repeated three times and performed in triplicate. The results are presented as means ± SD.

Results

*A **Pl**APP-related antigen is present and processed during sea urchin development*

The sea urchin larval nervous system consists in an array of neurons that control swimming and feeding and there begins to be present a more defined organization at late gastrula [18]. As a first approach to validate the sea urchin embryo as a model system in the study of Aβ-induced toxicity we planned to identify the presence of an antigen related to human APP. To investigate about the presence and expression of a *Pl*APP-related protein during sea urchin development, we carried out a Western blot analysis on protein extracts from embryos at gastrula stage and pluteus larvae at 48, 120 and 288 h of development.

After extraction, proteins were electrophoresed by SDS–PAGE and the corresponding immunoblot was incubated with an antibody against human APP. As shown in Fig. 1, a band of about 60 kDa is detectable in all the samples. Further, a band of about 10 kDa is present only in the extracts from 48 (pluteus stage), 120 and 288 h embryos. This result suggests that, after gastrula stage, a portion of the sea urchin *Pl*APP related protein is proteolitically cleaved producing a peptide, as occurs in higher organisms.

Moreover, to confirm the existence of the gene encoding for *Pl*APP, we cloned and sequenced a full-length cDNA (Accession No. FJ476254) by RT-PCR using total RNA from *P. lividus* blastula stage embryos.

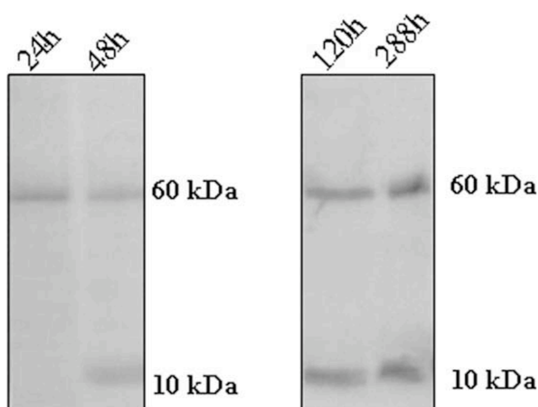

Fig. 1. *Pl*APP-related antigen is processed after 48 h of development. Same amount of proteins extracted from P. *lividus* embryos developed for 24 (24 h), 48 (48 h), 120 (120 h) and 288 (288 h) hours were loaded on a SDS–PAGE and the Western blot was incubated with anti-APP.

Localization of the PlAPP-related antigen

Some studies report that sea urchin nervous system is arranged in several clusters of neurons that urchin primordial nervous system is an array of neurons that has been successfully labelled by anti-serotonin [18]. To localize *Pl*APP-related antigen, *P. lividus* embryos were cultured for 48 and 96 h, to permit advanced morphogenetic nervous system events to occur. At these developmental stages the embryos were fixed and incubated both with anti-serotonin and anti-APP (Fig. 2). By immunohistochemistry using anti-APP we visualized a punctuate staining around the intestinal tube and oesophagus. When we merged this image on that obtained by using anti-serotonin, we detected a jellow staining, principally around the oesophagus, indicating that colocalization is occurred.

Next, we investigated whether the rAβ42, by moving through extracellular spaces, is able to be incorporated and deposited into the sea urchin embryos. Embryos treated with a mixture of rAβ42 assemblies, ranging from oligomers to large fibrillar aggregates, were fixed at 48 and 96 h of development and analysed by immunohistochemistry. For this experiment we utilized a lower rAβ42 concentration (0.6 μM) to avoid drastic damages in the embryos. Further, to be sure to detect exclusively the presence of exogenous rAβ42, we utilized anti-His antibodies against the 6-His tail which is linked to the N-terminus of the recombinant peptide [16]. As shown in Fig. 2 a punctuate staining, comparable to that obtained for APP and serotonin, is detectable indicating that rAβ42 is able to be internalized into the embryo, and suggesting that rAβ42 probably binds specific molecules.

rAb42 aggregates in fibrillar structures

We have previously demonstrated that rAβ42 induces toxicity in sea urchin embryos and oligomers are more toxic than aggre-

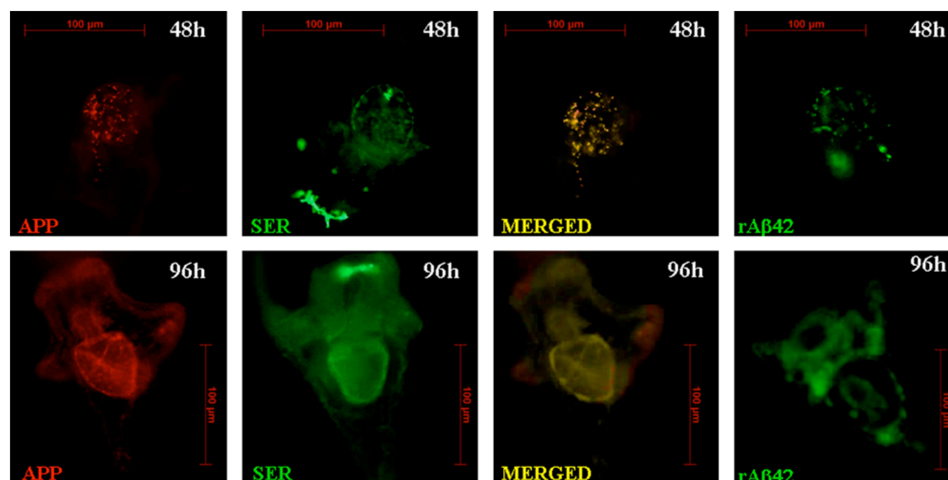

Fig. 2. Localization of *Pl*APP and esogenous rAβ42. *P. lividus* embryos at 48 and 96 h of development were fixed and localization of *Pl*APP-related antigen (48 h, APP), (96 h, APP) and serotonin (48 h SER, 96 h SER) was visualized by immunohistochemistry and utilizing anti-APP and anti-serotonin. The images were merged (48 h, MERGED), (96 h, MERGED). Two cell stage sea urchin *P. lividus* embryos were incubated with rAβ42 oligomers of different size and left to develop for 48 and 96 h (48 h, rAβ42), (96 h, rAβ42). The embryos were fixed and incubated with anti-His. Bar: 100 μm.

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gates [16]. To define a structure-cell death relationship for the Aβ-peptide, we investigated about a possible different effect for small oligomers and larger aggregates. As already described by dissolving rAβ42 at pH 7.2 and pH 3.1 we are able to obtain different species (oligomers or fibrillar aggregates) in solution as monitored by static and dynamic light scattering [16]. However, rAβ42 neutral and acid solutions were incubated for different times at 37 °C and their kinetic of aggregation was monitored after staining with thioflavin-T (ThT) (Fig. 3). No visible structures were detectable after staining of rAβ42 neutral solutions even after 4 days of incubation. In contrast, by monitoring rAβ42 acid solutions at different times, after 48 h larger and larger structures appeared until the formation of aggregates of dimensions of around 5 μm. In summary, for all the degenerative assays we utilized the rAβ42 neutral solution soon after their dissolution, and the rAβ42 acid solution after four days incubation.

rAβ42 induces apoptosis in sea urchin embryos

Small oligomers or fibrillar aggregates, were employed to investigate about the possibility that cell death occurs through the apoptotic mechanism. *Paracentrotus lividus* embryos at two cells stage were incubated with the two types of rAβ42 solutions (pH 7.2 and pH 3.1), described above, and the embryos were cultured until the controls arrived to gastrula and pluteus stages. In agreement to our previous results, we obtained a higher percentage of malformed or dead embryos upon treatment with rAβ42 oligomer forms with respect to the rAβ42 aggregate forms (pH 3.1) (data not shown). The survived embryos of both treatments were fixed and submitted to TUNEL assay. In contrast to the control embryos, showing only some stained nuclei probably due to physiological events, embryos treated with both oligomers and aggregates rAβ42 showed at 24 h of development a fair number of apoptotic

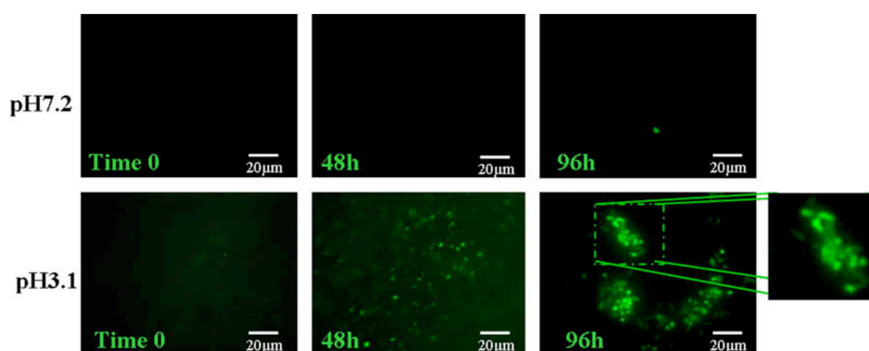

Fig. 3. Staining with ThT of oligomers and fibrillar aggregates. Kinetic of fibrillar aggregates formation of rAβ42 dissolved at pH 7.2 and pH 3.1. Staining with ThT at different times of incubation at $T = 37$ °C. Fluorescence images of representative areas of the observation field, immediately after dissolution (time 0) and after 48 (48 h) and 96 h (96 h) of incubation at $T = 37$ °C. A particular of aggregates at 96 h in shown in the inset. Bar: 20 μm.

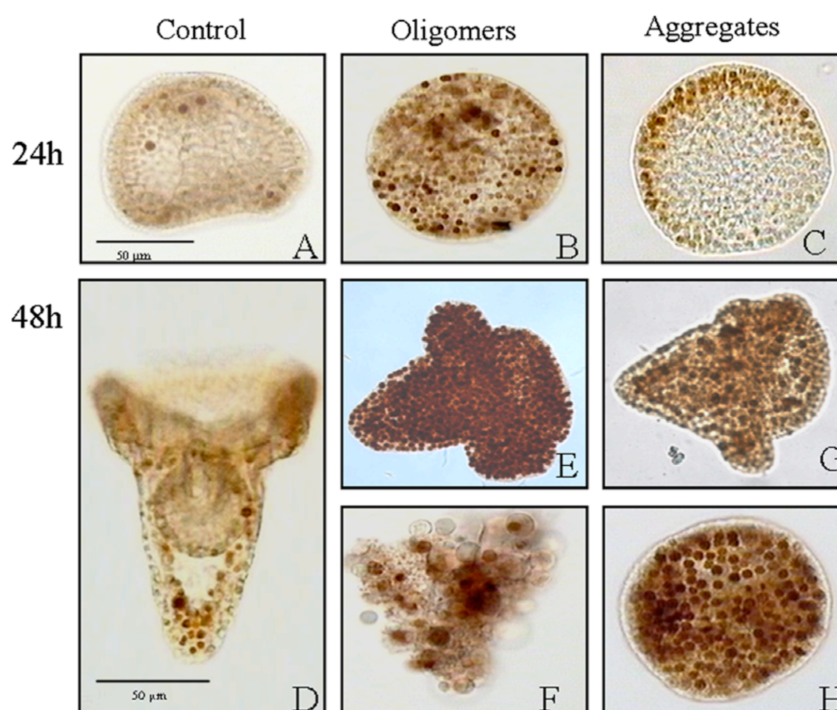

Fig. 4. Oligomers and aggregates activate apoptosis. Sea urchin embryos at 24 (24 h) and 48 (48 h) of development untreated (A and D) or treated with oligomers (B, E and F) or fibrillar aggregates (C, G and H), were fixed and submitted to TUNEL assay. Bar: 50 μm.

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nuclei (Fig. 4B and C), whereas at 48 of development almost all the nuclei of the survived embryos were stained. Difference in number of stained nuclei was observed between oligomers (Fig. 4E and F) and aggregates treated embryos (Fig. 4G and H), indicating that oligomers have more severe effects than aggregates.

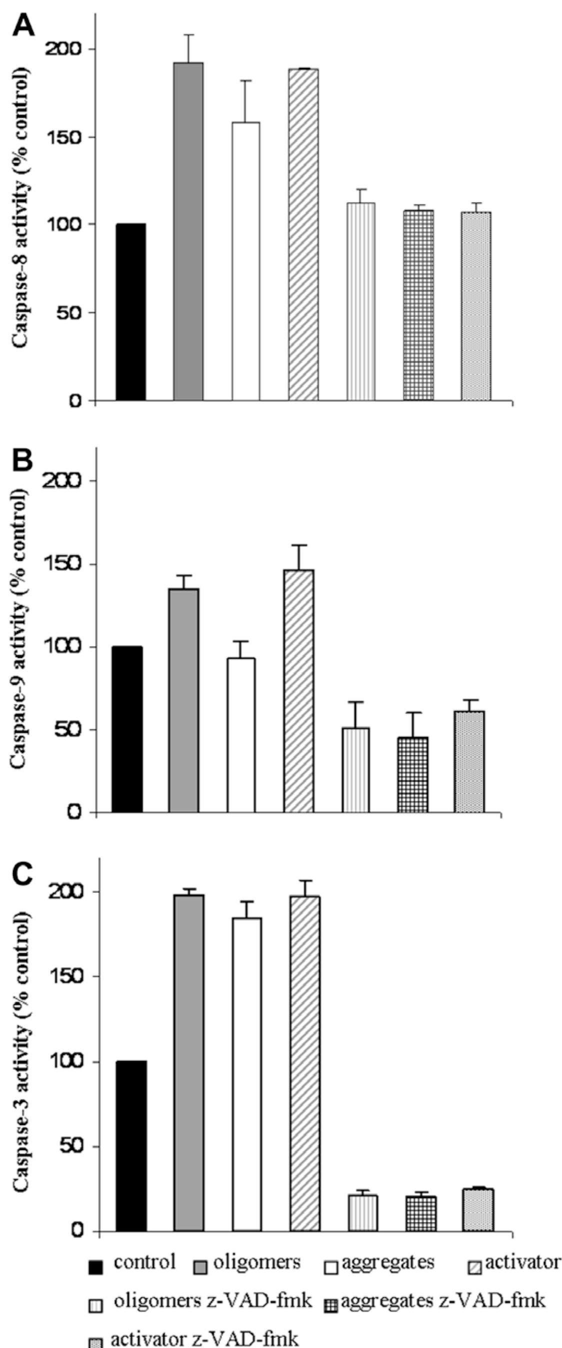

Fig. 5. Oligomers and fibrillar aggregates activate different caspases. *P. lividus* embryos at gastrula stage were treated with aggregates, oligomers or a caspase activator, without and with z-VAD-fmk and submitted to caspase 8 (A) or 9 (B) or 3 (C) luminescent assays. Inhibition of caspase 8 (A) and 9 (B) or 3 (C) activation was observed after incubation of *P. lividus* embryos with oligomers or aggregates or caspase activator plus z-VAD-fmk. As controls untreated embryos were utilized. On the left value expressed as a percentage of controls. The blank control values were subtracted from each. The data are means ± SD of three separate experiments, normalized respect to the control.

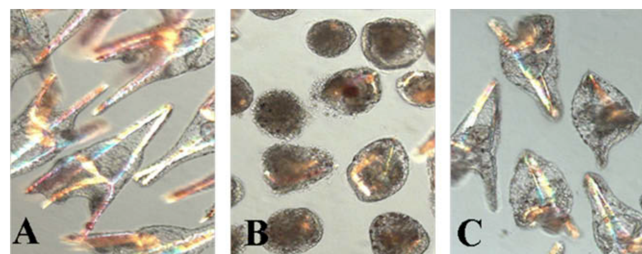

Fig. 6. z-VAD-fmk caspase inhibitor is able to rescue rAβ42 oligomers treated *P. lividus*. (A) untreated embryos (control) or treated with rAβ42 oligomers or with z-VAD-fmk together rAβ42 oligomers.

rAβ42 induces caspase-mediated apoptosis

Aβ induced apoptosis generally occurs through caspase dependent pathways even if caspase independent pathways have also been described [19,20]. In sea urchin embryos apoptosis is never found during cleavage stages. It begins to appear between early blastula and late gastrula stages [21]. On the basis of this knowledge we incubated *Paracentrotus lividus* at early gastrula stage with the two types of rAβ42 solutions (pH 7.2 and pH 3.1) and the embryos were cultured for 4 h.

Moreover, we investigate about the possibility that different apoptotic pathways, extrinsic or intrinsic, could be activated by the different rAβ42 aggregation forms. For this aim *P. lividus* embryos treated with oligomers and aggregates were submitted to caspase 8, 9 and 3 assays. A caspase activator and a caspase inhibitor z-VAD-fmk were employed as controls. Embryos treated with aggregates showed activation of caspase 8 (Fig. 5A) and no activation of caspase 9 with respect to the control (Fig. 5B), whereas embryos treated with oligomers showed both activation of caspase 8 (Fig. 5A) and of caspase 9 (Fig. 5B). *P lividus* embryos treated as described above were employed for the caspase 3 assay and, as expected, caspase 3 activity was detected for both treatments (Fig. 5C). Moreover, we treated *P. lividus* embryos both with rAβ42 oligomers and zVADfmk and after 48 h of development a rescue of the morphology was detected (Fig. 6C), with respect to the oligomers treated embryo (Fig. 6B), indicating that the drug has antagonised the amyloid effect.

Discussion

Reliable animal models are required to facilitate the understanding of neurodegenerative pathways in AD. They remain an invaluable tool for identifying molecular, cellular and pathological changes that trigger the onset of cognitive decline in AD. Furthermore, models should allow for the testing of compounds at various points of the pathogenetic cascade in order to search for disease modifying drugs. Here we propose the sea urchin *P. lividus* as an effective and simple model organism for studies of development and disease processes. Moreover, sea urchin occupies a key phylogenetic position as the only nonchordate detereustomes and the results obtained on this embryo can be extrapolated and compared to those of higher eukaryotes such as mammalian.

Here, we have used the sea urchin embryo to investigate about the degenerative pathway activated after a separate treatment with oligomers and fibrillar aggregates of a recombinant amyloid peptide. Employment of a recombinant Aβ peptide, with or without amino acids mutation, is an experimental approach that has been already employed to correlate structure and biological activity [22].

At the beginning to validate sea urchin as a model system for investigating about molecular mechanisms activated in AD, we ascertain the presence of a *Pl*APP-related antigen. This antigen is

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processed by gastrula stage, indicating that the production of a polypeptide is a conserved event for which, perhaps, presence of specific secretase is required. Similarly, a fragment of about 10 kDa is produced by α-secretase proteolitic cleavage of human APP, an alternative mechanism to Aβ formation that gives origin to the so called non-amyloidogenic pathway [4,23,24]. It will be interesting, in future, to investigate about the presence of secretases in sea urchin embryos, in particular of α-, β- and γ-secretase, the enzymes involved in human APP processing [23,24].

The presence of a *Pl*APP-like antigen, together with the localization of the rAβ42 in *P. lividus* primordial nervous system, is an encouraging data for our proposed aim, i.e., to utilize sea urchin to test cellular answer to Aβ treatment. It has been, indeed, reported that Aβ aggregates interact with their same precursor and this leads to a toxic gain of function, possibly by inducing an APP conformational change that triggers to cell death [24,25].

Neurodegeneration has been often associated to the programmed cell death mechanism, well known as apoptosis. Some studies have demonstrated an involvement of Aβ42 aggregates in activation of apoptosis and recent evidence have indicated that soluble protofibrils and small oligomers have toxic properties [26]. Thus, the toxicity is independent by the complexity of the aggregates, but perhaps differs by activating different intracellular or extracellular pathway, some of which can cross-talks among them, and induce degeneration.

Apoptosis in sea urchin development was investigated both for physiological events and for exposure to cytotoxic chemicals, heat stress, metals [21,27–29] or in response to UV radiation [30]. Here, we demonstrate that exposure to rAβ42 oligomers and fibrillar aggregates induces cell death by apoptosis and this event is mediated by caspases and reverted by a caspase inhibitor. Moreover, we demonstrated that aggregates employ exclusively extrinsic pathway, whereas oligomers both extrinsic and intrinsic pathways. This data suggests that a part of smaller soluble oligomers are able to penetrate into the cells and produce mitochondrial damage that activates caspase 9. Another part of oligomers could attach on neuron surface specific binding site and, perhaps, seed the aggregation process miming what in human pathology occurs. Moreover, the evidence that oligomers produce cell death by two apoptotic pathways could justify why, as previously demonstrated, oligomers result more toxic than aggregates [16]. Some evidences indicate that a mechanism of Aβ toxicity arises from its interaction with mitochondrial target such as Aβ-binding alcohol dehydrogenase (ABAD) [31–33]. Nevertheless, the possibility that small oligomers cross the membrane and penetrate into the intracellular environment could be in agreement with biophysical and modelling studies that have demonstrated the ability of Aβ to interact with a lipid bilayer due to its obliquity and hydrophobicity [34]. Moreover, some evidence indicates that extracellular Aβ contribute to the intracellular pool of Aβ, and this internalization occurs via an endocytic pathway involving caveolae/lipid rafts [35]. However, we cannot exclude that small oligomers, penetrating into the cells, produce stress to other intracellular organelles, such as endoplasmic reticulum. Identification of specific intracellular target for Aβ42 can be relevant to design therapeutic interventions aimed at preventing the neurotoxicity associated with β-amyloid protein.

Moreover, cell death signalling can start by the cell surface and we cannot exclude that especially rAβ42 aggregates, as well as neuritic plaques, trigger toxicity via membrane proteins. Recent reports indicate that monomeric and fibrillar forms of Aβ have the ability to bind to different membrane proteins and some of them have been identified [36,37]. As suggested, Aβ aggregates act as a membrane perturbant which alter the lipid bilayer and membrane-bound proteins or receptors, inducing activation of cell death pathway [38]. Thus, depending on the aggregation state, Aβ induced toxicity could occur through different paths and a simple model system can help, in future, to identify some of them. Further, in embryos exposed to z-VAD-fmk caspase activity was inhibited, indicating that *P. lividus* can provide a model to test the effectiveness of different compounds in offsetting the damage caused by Aβ42.

Finally, as transgenesis in *Drosophila*, *Caenorhabditis elegans* and zebrafish [39–43] have opened new possibilities in the screening of protein partners, modified genes and potential therapeutic molecules against AD, on the basis of the reported results, we retain that sea urchin can also be a good candidate for this kind of studies.

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