Dianxianning improved amyloid β-induced pathological characteristics partially through DAF-2/DAF-16 insulin like pathway in transgenic C. elegans

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Dianxianning (DXN) is a traditional Chinese formula, and has been approved in China for treating epilepsy since 1996. Here anti-Alzheimer’s disease activity of DXN has been reported. DXN improved AD-like symptoms of paralysis and 5-HT sensitivity of transgenic Aβ1-42 C. elegans. In worms, DXN significantly increased Aβ monomers and decreased the toxic Aβ oligomers, thus reducing Aβ toxicity. DXN significantly suppressed the expression of hsp-16.2 induced by juglone, and up-regulated sod-3 expression. These results indicated that DXN increased stress resistance and protected C. elegans against oxidative stress. Furthermore, DXN could significantly promote DAF-16 nuclear translocation, but it did not activate SKN-1. The inhibitory effect of DXN on the Aβ toxicity was significantly reverted by daf-16 RNAi, rather than skn-1 RNAi or hsf-1 RNAi. These results indicated that DAF-16 is at least partially required for the anti-AD effect of DXN. In conclusion, DXN improved Aβ-induced pathological characteristics partially through DAF-2/DAF-16 insulin like pathway in transgenic worms. Together with our data obtained by Morris water maze test, the results showed that DXN markedly ameliorated cognitive performance impairment induced by scopolamine in mice. All the results support that DXN is a potential drug candidate to treat Alzheimer’s diseases.

Alzheimer’s disease is a progressive neurodegenerative disorder which is becoming more prevalent in ageing populations worldwide. It is neuropathologically characterized by extensive neuronal loss and the presence of neurofibrillary tangles and senile plaques. Nowadays over 46 million people suffer from AD in the world, and it is estimated to increase to 131.5 million by 2050. So far, there are only five FDA-approved anti-AD drugs on the market. Unfortunately, these drugs can only delay the onset of dementia, and none of them can halt or reverse the disease. Those medications for treating AD have never met the medical need, it is urgent to find effective and safe drugs against AD.

A lot of evidence have shown that the pathological hallmarks of AD are included Aβ aggregation and deposition with senile plaque appearance, tau hyper phosphorylation with tangle formation, which destroy synapses, induce brain inflammation, eventually lead neuronal death and severe brain shrinkage. Among them, the Aβ is generated in brain from the amyloid precursor protein (APP) after sequential proteolytic cleavage by β-secretase and γ-secretase enzymes. Under normal conditions, the produced Aβ is quickly removed from the brain. However, the mutations that increase Aβ production and the factors that decrease Aβ clearance or enhance Aβ aggregation will lead to Aβ self-aggregates into assemblies ranging from oligomers to protofibrils, fibrils and amyloid plaques. Aβ cascade hypothesis of AD is widely accepted. Accordingly, the anti-AD therapies primarily aim to lower the level of Aβ generation, aggregation or accumulation in the brain of AD patients. However, many of such drug candidates were demonstrated to manifest only limited benefit in AD patients. The reason is mainly explained by that other complex pathogenic cascades of related Aβ induced toxicity, are not affected, thereby the
amyloid plaques in APP/PS1 transgenic mice and effectively promote their learning memory behavior. The link between these two disorders. In fact, antiepileptic drugs have been involved in preclinical studies or discovery resources for treating AD.

The Dianxian Ning (DXN) has been approved in China Food and Drug Administration as an antiepileptic drug since 1996. DXN consists of eight Chinese medicine herbs, namely Valeriana jatamansi Rhizoma et Radix, Rhi zoma Acori tatarinowii, Ramulus Uncariae cum Uncis, Semen Pharbitidis, Semen Euphorbiae, Radix et Rhizoma Valeriana officinalis, Rhi zoma et Radix Nardostachys, and menthol crystal. According to the theory of traditional Chinese medicine (TCM), DXN can clinically eliminate phlegm for resuscitation, stop wind, and calm the spirit. Previous work reported that Valeriana jatamansi Rhizoma et Radix possesses anti-inflammatory, anti-oxidant activities. In fact, TCM similarly function on a multi-targeted manner to treat the symptoms. Previous studies have indicated that AD patients have an increased risk of developing epileptic seizures, and the risk of epileptic activity is highest in those patients with early-onset dementia who over-express amyloid precursor protein (APP) and Aβ. Epidemiological data indicates that patients with AD and seizure disorders have greater cognitive impairment, faster progression of symptoms, and more severe neuronal loss at autopsy than those without seizures. There is a close relationship between epilepsy and AD, and Aβ has been identified to be the link between these two disorders. In fact, antiepileptic drugs have been involved in preclinical studies or clinical trials for treating AD. Levetiracetam can suppress neuronal network dysfunction and reverse AD damage and cognitive impairment in mice. Lamotrigine can attenuate deficits in synaptic plasticity and accumulation of amyloid plaques in APP/PS1 transgenic mice and effectively promote their learning memory behavior. Valproic acid can inhibit Aβ generation, neuritic plaque production, ameliorate cognitive performance in AD mice, but it is unlikely to affect patient cognitive function. Anyhow, repurposing antiepileptic drugs is still available to discover potential anti-AD drug candidates.

Caenorhabditis elegans is an inexpensive tool to be widely used to evaluate anti-AD drug candidates based on Aβ hypothesis. In this study, we investigated the effect of DXN on Aβ-induced injury using transgenic C. elegans model which exhibits several pathological behaviors due to Aβ toxicity. We also examined whether DXN can improve cognitive performance impairments induced by scopolamine in mice by Morris water maze test. Finally, we explored the underlying mechanism that DXN exerted its possible anti-AD action. Our results provide evidences for that DXN as a traditional Chinese anti-epileptic medicine is likely to be a potential drug candidate to fight against AD. Also, our results provide clues to introduce anti-epileptic TCM as potential drug candidate discovery resources for treating AD.

Results

DXN ameliorated AD-like symptoms induced by Aβ1-42 expression. To determine whether the DXN can protect against the toxicity induced by Aβ1-42 expression, transgenic C. elegans smg-1 (cc546)I temperature sensitive Aβ strain CL4176 was used, and human Aβ1-42 peptide has been transfected into worms and controlled by the promoter of myo-3, thus the over expression of Aβ1-42 in the muscle cells can induce AD-like symptom of Aβ-dependent paralysis. We found that DXN significantly delayed worm paralysis in a dose-dependent manner (Fig. 1A).

Serotonin (5-HT) is an important neurotransmitter that regulates worm behaviors of locomotion, egg-laying, olfactory learning and mating. Exogenous 5-HT can inhibit C. elegans locomotion, leading active worm into...
altered exogenous gfp gene expression (Fig. 3D,E), suggesting that DXN and Vj treatment possible inhibiting [smg-1 and it should not be reduced. Additionally, transgenic worm in anti-AD activity of DXN and Vj for delaying paralysis. Anyway, the effect of Vj largely decreased than that of to a less degree (Fig. 3C). Of course, shorter dosing time and less DXN and Vj entry into worms led the decline animals were on the same development stage. The results showed that DXN and Vj did delay worm paralysis, but that Vj was the principal component of DXN. It is deserved to notice that Vj alone also significantly shortened expression, and at that time worm body length, but DXN did not (Fig. 3B). In contrast to drug treatment from eggs, worms were adminis-

Table 1. Comparison of average escape latency time for each group in the hidden platform trial. Data are the average of 14 animals in each group. There is significant difference among these groups when symbols are different (P < 0.05).

| Treatment       | Escape latency time/second |
|-----------------|-----------------------------|
|                 | 1st day | 2nd day | 3rd day | 4th day | 5th day |
| Control         | 58.37 ± 3.25 | 33.60 ± 4.45 | 29.76 ± 2.96 | 21.36 ± 4.19 | 16.54 ± 2.72* |
| Model           | 45.06 ± 1.90 | 42.64 ± 1.87 | 32.68 ± 3.93 | 33.28 ± 2.72 | 34.18 ± 2.68* |
| 0.5 g/kg Piracetam | 41.01 ± 2.62 | 33.02 ± 3.04 | 29.83 ± 2.79 | 28.06 ± 3.33 | 21.07 ± 2.61** |
| 0.39 g/kg-DXN   | 35.77 ± 3.65 | 31.08 ± 3.63 | 33.11 ± 3.51 | 27.61 ± 3.63 | 25.75 ± 3.22** |
| 2.35 g/kg-DXN   | 43.86 ± 3.29 | 35.43 ± 3.08 | 34.29 ± 3.66 | 27.87 ± 3.05 | 20.71 ± 3.14** |

paralysis. C. elegans snb-1/A\(\beta_{1-42}\) strain CL2355 shows serotonin hypersensitivity when A\(\beta\) are over-expressed in nerve system22,23. Here we investigated whether DXN treatment could reverse serotonin hypersensitivity induced by the toxicity of A\(\beta\) to nerve cells in the transgenic snb-1/A\(\beta_{1-42}\) worms. Our results showed that snb-1/A\(\beta_{1-42}\) worms were hypersensitive to exogenous 5-HT, but CL2122 without A\(\beta\) expression in nerve cells were not. DXN significantly alleviated the paralysis symptom of hypersensitive response to 5-HT in a dose-dependent manner (Fig. 1B).

Scopolamine is an anti-cholinergic agent to induce aspects of the memory loss and cognitive impairment, which resemble the hallmark symptoms observed in AD associated with cholinergic dysfunction at least on some degree. The scopolamine model has been widely used for screening possible AD symptom therapeutic agents to improve cognitive performances24. In present work, this model was used to evaluate the effects of DXN on learning and memory impairment by the Morris water maze test. Compared with the normal control group, the memory training results indicated that the mean escape latencies were significantly lengthened in scopolamine treated mice on the 5th day of the experiment. DXN improved learning and memory performance of mice as piracetam (Table 1). In probe test, the swimming time in the target quadrant of DXN treated animals significantly lengthened in contrast to the model group (Fig. 2A). The platform frequency remarkably increased in DXN and piracetam treated groups (Fig. 2B). Compared to model group, the length of swimming path between drug treatment groups and normal control group were not significant in probe test (Fig. 2C). These results suggested that DXN indeed promoted learning and memory performance in scopolamine treated mice.

**DXN exerted anti-AD like action by its principal component Valeriana jatamansi Rhizoma et Radix.** DXN is a kind of traditional Chinese medicine, and it is composed of eight herbs. Previous works have suggested that three herbs of Valerianae jatamansi Rhizoma et Radix (Vj), Rhizoma Acori tatarinowii (Ra) and Ramulus Uncariae cum Uncis (Ru) in DXN have anti-AD activity1,14–17. In present work, Vj, Ra and Ru at an equivalent dose in DXN were firstly used to tested whether they can ameliorate AD-like symptom of delaying paralysis in worms. We further tried to investigate that whether DXN can be reduced to one or more herbs which can function as the principal component, and finally some effective compounds based anti-A\(\beta\) therapy for treating AD can be extracted from the right principal herbs.

The results showed that Vj delayed worm paralysis as DXN complete formula to a similar degree. Ra and Ru significantly alleviated the AD-like symptom in worms to a much lesser degree (Fig. 3A). These results supported that Vj was the principal component of DXN. It is deserved to notice that Vj alone also significantly shortened worm body length, but DXN did not (Fig. 3B). In contrast to drug treatment from eggs, worms were adminis-

| Treatment       | Escape latency time/second |
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| 0.5 g/kg Piracetam | 41.01 ± 2.62 | 33.02 ± 3.04 | 29.83 ± 2.79 | 28.06 ± 3.33 | 21.07 ± 2.61** |
| 0.39 g/kg-DXN   | 35.77 ± 3.65 | 31.08 ± 3.63 | 33.11 ± 3.51 | 27.61 ± 3.63 | 25.75 ± 3.22** |
| 2.35 g/kg-DXN   | 43.86 ± 3.29 | 35.43 ± 3.08 | 34.29 ± 3.66 | 27.87 ± 3.05 | 20.71 ± 3.14** |

further evaluated compound 1–15 extracted from Vj (Fig. 4), and treated worms with each compound at relative final concentration due to their different maximum saturated solubility in DMSO. In contrast to Vj, almost all of them delayed worm paralysis induced by over-expression of A\(\beta\) to a much lesser degree, except that 100 \(\mu\)M of compound 15 did not have any anti-AD activity, 250 \(\mu\)M of compound 11 significantly promoted worm paralysis (Fig. 5). It is deserved to notice that compound 6 is baldri nal, which is regarded as the marker substance of Vj and DXN, and its content is 0.930 mg per gram of DXN or Vj coarse powder. In Fig. 5, 160 \(\mu\)M baldri nal was at a dosage higher by 2.6-fold than that contained in Vj used. Previous work showed that an iridoid, 10-O-trans-p-coumaroylcatalpol at similar dose or less significantly inhibits \(\alpha\)-synuclein aggregation in worms26. So that, it cannot be excluded that more active compound will be isolated from Vj in the future work. Because relative large amount of baldri nal is contained in Vj, its anti-AD activity is still dissecting in our lab. At present, it is reasonable to believe that DXN needs complete Vj.

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DXN reduced Aβ oligomers rather than amyloid deposits in transgenic C. elegans. To explore whether the DXN affects the aggregation of the large Aβ species, unc-54/human Aβ1-42 worm strain CL2006 that constitutively over expresses human Aβ1-42 in muscle cells was used. Although memantine is clinically used as a selected NMDA receptor antagonist, evidence recently indicates that it lowers the Aβ level and decreases Aβ plaques to support memantine may be a disease-modifying drug for treating AD 27–30. Therefore, memantine has been used as a positive control. We found the DXN had no remarkable effect on the deposits of the Aβ1-42 peptide, suggesting that the DXN could not decrease the aggregation of the Aβ species significantly (Fig. 6A,B). As expected, memantine significantly lowered Aβ deposits (Fig. 6A,B). Considering the current evidence shows that soluble Aβ oligomers, rather than senile plaques (SPs) or Aβ fibrils, are the toxic species to link to...
Figure 4. Compounds of 1–15 extracted from *Valeriana jatamansi*: 1: 8-hydroxyl-pathcouli alcohol; 2: 8-acetoxy-pathchouli alcohol; 3: Ursolic acid; 4: 3β- Hydroxy-5α,8α-epidioxyergosta-6,22-diene; 5: stigmast-5-ene-3β-ylformate; 6: baldrinal; 7: (-)-bornyl ferulate; 8: vanillin; 9: (3S, 4S, 5S, -7S, 8S, 9S)-3,8-ethoxy-7-dihydroxy-4,8-dimethylperhydro cyclopenta - [c] pyran; 10: valerol A; 11: salicylic acid; 12: (4β,8β)-8-ethoxy-3-methyl-10-methylene-2,9-dioxatricyclo[4.3.1.03,7]decan-4-ol; 13: Valeriananoids D; 14: 5-(hydroxymethyl)-2-furfuraldehyde; 15: 8-methylvaleropiate.

Figure 5. The effects of compound 1–15 extracted from DXN on AD-like symptoms in the temperature sensitive Aβ worms. Data are the average of three replicates with about 180 worms in each group. ***Indicated that there was significant difference between treatment group and control group at P < 0.001.
neurodegeneration and cognitive decline\textsuperscript{31,32}, we performed the Western blotting analyses to test whether the DXN can reduce the toxic Aβ oligomers. Primary antibody of 6E10 against Aβ was used in western blotting analysis, and the relative densities of Aβ monomer of 4 kDa band and higher molecular weight oligomer of 20 kDa band were quantified\textsuperscript{22}. The result showed that DXN significantly reduced the level of Aβ oligomers and increased the level of Aβ monomers (Fig. 6C,D). It indicated that DXN could significantly change the Aβ oligomers with high neurotoxicity into Aβ monomers, which is much less toxic than Aβ oligomers.

**DXN delaying paralysis of AD worms was partially mediated by DAF-16 activation.** DAF-16 is a crucial component of DAF-2 insulin like pathway to regulate many gene expressions for defending against various stresses including oxidative stress and proteotoxicity\textsuperscript{33}, its activation has been proved to be benefit for delaying paralysis induced by human Aβ excessive expression in the muscle in *C. elegans*\textsuperscript{34}. To examine whether DAF-16 is required for DXN protecting worms from severe stress of aberrant Aβ generation and accumulation, we knocked down expression of the transcription factor DAF-16 by RNAi, and found that the inhibitory effect of DXN on worm paralysis significantly decreased (Fig. 7A). We further constructed a *daf-16* mutant strain under genetic background of the temperature sensitive Aβ strain CL4176 by standard genetic crosses. Treating the AD worms with *daf-16* mutation, a similar result has been obtained (Fig. 7B). These results indicated that DXN reduced Aβ toxicity at least partially through a DAF-16-based mechanism. After DXN treatment, DAF-16 was indeed activated and displayed nuclear translocation from the cytosol to the nucleus (Fig. 7C,D). Further, its typical downstream target SOD-3 was markedly up-regulated (Fig. 8A,B). According to our knowledge, DAF-16 activation increases stress resistance and then leads to lengthen lifespan. Expectedly, the result of lifespan assay on *unc-54/human Aβ*1-42 worm strain CL2006 treated with DXN showed that DXN did significantly extend worm lifespan (Fig. 3S).

As a chaperon, HSP-16.2 can be induced by the abnormal Aβ proteins to increase their sequestration, degradation, and refolding\textsuperscript{35}. In our experiment, the expression of *hsp-16.2* was examined after DXN pretreatment by using TJ375. The transgenic worm strain TJ375 has a GFP reporter gene controlled by the promoter of *hsp-16.2*, it exhibits an excessive GFP expression in the pharynx after exposure to oxidative stress such as juglone. The results showed that DXN alone did not significantly affect the *hsp-16.2::gfp* expression. However, when *C. elegans* were pretreated with DXN and further administrated with juglone, the fluorescence density in the pharynx of worms under juglone-induced oxidative stress significantly decreased compared to the DXN untreated group (Fig. 8C,D). We further treated AD worms with RNAi for *sod-3* and *hsp-16.2* (Fig. 8E). The results showed that *sod-3* RNAi significantly promoted worm paralysis suggesting that SOD-3 is required for battling against Aβ toxicity. It is seemed to find that DXN delayed AD-like symptoms to the same degree as DXN treatment alone without *sod-3* RNAi, but it did not indicate that SOD-3 was not required for DXN-mediated neuroprotection.
Possibly, the compensatory upregulation of other sod genes or related detoxifying signaling pathway counteracted the effect of sod-3 RNAi on DXN-mediated delaying worm paralysis. As for hsp16.2 RNAi, no obvious effect was observed. It might be explained by that hsp-16.2 is only expressed under stress.

Transcription factor SKN-1 modulates genes that fight against oxidative and xenobiotic stress and genes that regulate protein homeostasis. After skn-1 RNAi, the Aβ-induced paralysis phenotype in the temperature sensitive Aβ strain CL4176 was still significantly delayed by DXN treatment (Fig. 1S-A), indicating that SKN-1 was not involved in the protective effect of DXN. It was further validated by SKN-1 did not appear nuclear translocation at all (Fig. 1S-B).

HSF-1 regulates many gene expressions in response to stress, and has been recognized to control disaggregation and degradation of Aβ. In present work, after hsf-1 RNAi, the Aβ-induced paralysis phenotype in the temperature sensitive Aβ strain CL4176 was still significantly delayed by DXN treatment (Fig. 1S-C), indicating that HSF-1 was not involved in the protective effect of DXN. In present work, RNAi for hsf-1 and skn-1 significantly promoted worm thermal stress sensitivity (Fig. 1S-D). Therefore, the lack of effect of RNAi on worm paralysis after DXN treatment could not result from that the RNAi treatment did not work.

Discussion

DXN has been clinically used to treat epilepsy in China since 1990s. In present study, it strongly ameliorated Aβ-induced paralysis and 5-HT hypersensitivity in the transgenic C. elegans in a dose dependent manner (Fig. 1). Furthermore, DXN significantly alleviated learning and memory impairment induced by scopolamine in mice (Fig. 2). Those results indicated that DXN was potential to serve as a drug candidate for treating AD. In fact, we also tested the anti-AD activity of other two traditional Chinese formulas of Dianxianping tablet (DXP) and Guishaozhenxian tablet (GS), these two formulas also have been approved by CFDA for treating epilepsy. Our results showed that DXP and GS are less effective in comparison to DXN at their concentration available (Fig. 2S), and both of them could not be used on a larger dosage due to that they have severely delayed worm growth and development at their concentration in present work. Therefore, although there is close link between epilepsy and AD, whether an anti-epileptic drug can be used for treating AD still needs to be carefully investigated in details.

In present work, Vj has been proved to exert its delaying paralysis of AD-like symptom as a principal component (Fig. 3A). Further, compounds of 1–15 isolated from Vj significantly delayed worm paralysis and have anti-AD activity on a certain degree, except that compound 15 did not affect worm paralysis and compound 11 even aggravated AD-like symptom. In comparison with the complete herb of Vj, they only exhibited moderate protective effect (Fig. 5). Similarly, coffee extract can protect worms from Aβ toxicity, whereas caffeine does only...
...to a much lesser degree\textsuperscript{29}. Previously, Vj has been demonstrated to have anti-inflammatory and anti-oxidant activity\textsuperscript{14, 15}. Several other compounds of iridoids, sesquiterpenoids extracted from this species and other related species of genus \textit{Valeriana} have been shown to have neuroprotective effects\textsuperscript{25, 39}. It is reasonable to conclude that these compounds can exert additive or synergistic effect for anti-AD. Of course, it cannot be excluded that more active compound will be isolated from Vj in the future work.

DXN, as a kind of traditional Chinese medicine, is composed of eight herbs. Among the herbs, besides Vj, Ra and Ru have anti-AD activity\textsuperscript{1, 14–17}. Our results show that Ra and Ru did not ameliorate AD-like symptom in worms additively or synergistically (Fig. 3A), but complete DXN did not delay nematode growth any more (Fig. 3B), indicating that DXN is less toxic than Vj alone. It is especially important for a potential anti-AD drug to act effectively to be used just at asymptomatic and preclinical stage, and the disease manifests its clinical symptoms after a decade or more, thus anti-AD therapy being a long term prevention\textsuperscript{40}. The present evidence supported that DXN could not be reduced. Further work needs to investigate on the actions of these herbs through a feasible AD model \textit{in vivo} or \textit{in vitro} based on other respects of the pathogenesis of this devastating disease.

After DXN treatment, A\textsubscript{β} oligomers significantly reduced, but A\textsubscript{β} monomers increased in AD worms (Fig. 6C, D). It is well recognized that A\textsubscript{β} oligomers induce the death of neurons and are responsible for AD-related memory loss. Moreover, A\textsubscript{β} oligomers are closely related to severity of dementia in AD patients\textsuperscript{31, 39}. Accumulating evidence suggests that A\textsubscript{β} oligomers but not A\textsubscript{β} monomers or A\textsubscript{β} deposits are correlated with A\textsubscript{β} toxicity\textsuperscript{41, 42}. Our results supported that DXN strongly ameliorating A\textsubscript{β} toxicity in the transgenic \textit{C. elegans} over-expressed human A\textsubscript{β}\textsubscript{1–42} might be a consequence of its directly or indirectly promoting the shift from the toxic A\textsubscript{β} oligomer to less-toxic monomer form.

Small HSPs are Low-molecular-weight Heat Shock Proteins (12–43 kD) in response to a series of injuries including thermal stress and oxidative stress\textsuperscript{43}. Moreover, small HSPs also participate in preventing the accumulation of several different types of toxic proteins such as A\textsubscript{β} and ployQ\textsuperscript{44}. HSP16.2 can suppress A\textsubscript{β} toxicity by assisting abnormal protein sequestration, degradation, and refolding in AD \textit{C. elegans}\textsuperscript{35}. Here, DXN treatment did not increase the expression of hsp-16.2 (Fig. 8A), indicating that toxic A\textsubscript{β} oligomer reduction after DXN treatment was not mediated by molecular chaperon HSP-16.2. Then whether DXN can directly bind to A\textsubscript{β} proteins to inhibit their aggregation and increase their less toxic monomers needs further investigated. However, DXN counteracted the up-regulation of hsp-16.2 induced by juglone (Fig. 8C, D). Since juglone is a reactive oxygen species generator\textsuperscript{45}, it gave us a notion that DXN could reduce A\textsubscript{β} toxicity through anti-oxidant activity. It agrees with the previous work that components of DXN, Vj and \textit{Rhizoma Acori tatarinowii} possess high antioxidant activity\textsuperscript{14, 36, 46}.

Figure 8. SOD-3 was required for DXN inhibiting A\textsubscript{β} toxicity. (A) sod-3 expression was up-regulated after 15 mg/mL DXN treatment, and worms were treated with 20 mM juglone as positive control. The scale bar was 40 μm. (B) Quantification of sod-3 expression. (C) 15 mg/mL DXN inhibited the expression of Phsp-16.2::GFP induced by 40 μM juglone, and worms treated with 20 mM juglone were used as positive control. The scale bar was 25 μm. (D) Quantification of the expression of hsp-16.2. In (A–D), data are the average of three replicates with about 90 worms in each group. There is significant difference among these groups when symbols are different (P < 0.05). (E) The effects of sod-3 RNAi and hsp16.2 RNAi on DXN inhibiting worm paralysis induced by A\textsubscript{β}\textsubscript{1–42} over-expression. Data are the average of three replicates with about 120–180 worms in each group. ** Indicates that there was significant difference between treatment groups and control group at P < 0.001.
E. coli on solid nematode growth medium (NGM) seeded with standard food resource of β oligomers and increased less toxic by DAF-16 activation in worms. DXN also directly or indirectly reduced A inclusion, as shown in Fig. 9, DXN suppressing A (Fig. 1SA,B). Additionally, SKN-1 did not exhibit nuclear translocation after DXN treatment (Fig. 1SC). In conclusion, as shown in Fig. 9, DXN suppressing A3-induced pathological behaviors was at least partially mediated by DAF-16 activation in worms. DXN also directly or indirectly reduced A3 oligomers and increased less toxic A3 monomers.

Materials and Methods
Preparation of DXN. DXN is composed of eight herb drugs at dose of 500 g Valerianaec jatamansi Rhizoma et Radix (Vj), 200 g Semen Pharbitidis, 200 g Rumulus Uncariae cum Uncis (Ru), 500 g Rhizoma Acori tatarinowii (Ra), 200 g Rhizoma et Radix Nardostachyis, 15 g Semen Euphorbiae, 0.3 g Menthol, 0.62 g Radix et Rhizoma Valeriana officinalis. DXN was prepared according to WS3-B-2823-97 in Drug Standard issued by Ministry of public health, China (Fourteenth prescriptions of Chinese Medicine). Briefly, herb drugs were crushed into coarse powder and extracted by diacolation and decoction methods. Among them, 200 g Vj coarse powder without extraction and extraction with water, centrifuged at 10,000 rpm for 10 min, the resultant supernatant containing DXN at a concentration of 150 mg/mL as a stock solution was stored at 4 °C before use.

C. elegans strains and maintenance. The wild-type C. elegans N2, transgenic C. elegans CL2355 [snb-1(A)/AJ1-42/long 3′-UTR + mtl-2::GFP]; CL2122 dvIs15 [(pPD30.38) unc-54(vector) + (pCL26) mtl-2::GFP]; CLA176 smg-1(cc546); dvIs27 [myo-3::Aβ(1-42):let-851 3′UTR) + rol-6(su1006)]; CL2006, dvIs2 [pCL12 (unc-54/human Aβ peptide1-42 minigene) + prF4]; CL2179, smg-1(myo-3::GFP); TJ356 [zIs356 (Pdaf-16::daf-16a/b::GFP + rol-6)]; TJ375 [gpl11(sod-3::GFP)]; CF153 [muls84 (pAD76 sod-3::GFP + rol-6)]; LG333 (gel5(skn-1b::GFP)]; CF1038 [daf-16(mu86) I] were obtained from Caenorhabditis Genetics Center (CGC) (University of Minnesota, Minneapolis, MN). All worms were propagated at 20 °C except CLA176 at 16 °C on solid nematode growth medium (NGM) seeded with standard food resource of E. coli OP50.

Paralysis assay. Transgenic C. elegans of temperature sensitive A3 strain (CL4176) maintained at 16 °C were egg-synchronized on the NGM plates containing with 0, 1 mg/mL, 5 mg/mL, 15 mg/mL DXN. Other principal herbs of Vj, Ra and Ru were used at an equivalent dose as they were in DXN, and 3.2 mg/mL Vj, 3.7 mg/mL Ru and 0.6 mg/mL was used, respectively. For compounds of 1–15 extracted from Vj, they were all diluted at indicated final concentrations in 0.1% DMSO according to their maximum saturated solubility. Compounds of 1–7 were relatively used at a final concentration of 50 μM, 100 μM, 20 μM, 20 μM, 160 μM, 120 μM, compounds of 8–11 were used at 250 μM and compounds of 12–15 were used at 100 μM. Worms were induced to express human Aβ1-42 till they were at L3 stage larvae by up-shifting the incubation temperature from 16 °C to 25 °C for 34 h. The transgenic worms were scored at 2 h intervals for paralysis till all animals in negative control group were paralyzed.

Figure 9. Schematic diagram of DXN action on suppressing A3 toxicity. DXN directly or indirectly reduced Aβ oligomers and increased less toxic Aβ monomers, further, DXN induced DAF-16 activation to regulate its downstream stress responsive gene expressions, such as sod-3, hsp-16.2, at least partially to exert its anti-AD activity.
performed as described previously. Briefly, worms were collected by washing with M9 and were fixed in 4% paraformaldehyde in PBS, pH 7.4, at 4 °C for 24 h. The fixative solution was replaced by permeabilization solution (0.1% Triton-X 100, 5% BSA) for 1 h at room temperature. Blotting was carried out overnight at 4 °C. A secondary antibody. Worms were considered to be paralyzed if they did not move at all or only moved their heads.

**Fluorescence staining of Aβ deposits.** Transgenic *C. elegans* with unc-54/human Aβ1-42, maintained at 20 °C were egg-synchronized onto NGM plates and grown until the L4 stage, worms were then moved to fresh NGM plates containing 0, 1 mg/mL, 5 mg/mL, 15 mg/mL DXN. Two days later, Thioflavine-S (ThS) staining was performed as described previously. Briefly, worms were collected by washing with M9 and were fixed in 4% paraformaldehyde in PBS, pH 7.4, at 4 °C for 24 h. The fixative solution was replaced by permeabilization solution (0.1% Triton-X 100, 5% BSA) for 1 h at room temperature. Blotting was carried out overnight at 4 °C. A secondary antibody.

**Exogenous serotonin sensitivity assay.** After egg-synchronized, the transgenic snb-1/Aβ1-42 worm were placed at 20 °C on NGM plates seeded with OP50 for 48 h. The worms were treated with 0, 1 mg/mL, 5 mg/mL, 15 mg/mL DXN for another 48 h, respectively. 30 worms in each group were washed with M9 buffer for three times and were transferred into 200 μL M9 buffer containing 1 mg serotonin (Sigma), paralyzed worms were scored after 5 min. Animals were considered to be paralyzed if they did not move at all within 5 sec. Worm strain CL2122 without Aβ expression in nerve cells was used as a transgenic control.

**Western blotting.** Human Aβ1-42 in the temperature sensitive Aβ strain (CL4176) was identified by immuno-noblotting on a Tris-Tricine gel by the standard Western blotting assay. Wild type N2 was used as negative control of the expression of Aβ. Worms were treated as paralysis assay with or without 15 mg/mL DXN. Nematodes were then washed three times in M9 to remove the bacteria after incubation temperature up-shifting for 40 h and finally harvested in distilled water with protease inhibitor cocktail (Sigma, P2714) added, quickly frozen and stored at -80°C. Worms were boiled in sample lysis buffer (1× protease inhibitor cocktail, 62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 4% β-mercaptoethanol) for 10 min, then separated on ice to cool, centrifuged at 14,000 g for 5 min. Total protein content in the supernatant was measured by Lowry method. Proteins were boiled for 5 min in sample loading buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 4% β-mercaptoethanol, 0.0005% bромophenol blue). Samples were performed electrophoresis on the Tris-Tricine gel with 70 μg total protein in each lane. After the gel was transferred to PVDF membrane, the membrane was boiled in PBS for 15 min before blocking with a solution of 5% milk in TBS-Tween (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Tween-20) for 1 h at room temperature. Blotting was carried out overnight at 4 °C. Aβ proteins were detected by 6E10 (Biologend, 803001) at 1:1000 dilution. Internal control of β-tubulin was probed by polyclonal antibody against it (Sigma, T6199) at 1:1000 dilution. Goat anti-mouse HRP-labelled IgG was used as a secondary antibody.

**Nuclear localization of DAF-16.** Worm *daf-16::gfp* (TJ356) expressing GFP as a reporter was used for detecting the intracellular distribution of DAF-16. Synchronized L1 larvae worms were fed with or without 15 mg/mL DXN for 72 h. Subsequent to this treatment, worms were mounted on glass slides and their nuclear localization of DAF-16 were observed under a fluorescence microscope (BX53; Olympus, Japan). Worms are scored based on two categories of cytosolic and nuclear distribution with respect to the major localization of the DAF-16::GFP fusion protein. DAF-16 intracellular location was indicated by the ratio of nuclear translocation in the experimental population.

**RNA interference (RNAi).** RNAi gene expression clones were constructed as described by Fraser et al. Briefly, PCR products were synthesized using Q5 High-Fidelity DNA Polymerase (New England Biolabs, USA) with genomic DNA as template by relative primers listed in Table 2, and then inserted into L4440 vector (Addgene plasmid 1654). Recombinant plasmids were subsequently transformed into the *E. coli* HT115 (DE3) bacterial strain (CL4176) was identified by immuno-noblotting on a Tris-Tricine gel by the standard Western blotting assay. Wild type N2 was used as negative control of the expression of Aβ. Worms were treated as paralysis assay with or without 15 mg/mL DXN. Nematodes were then washed three times in M9 to remove the bacteria after incubation temperature up-shifting for 40 h and finally harvested in distilled water with protease inhibitor cocktail (Sigma, P2714) added, quickly frozen and stored at -80°C. Worms were boiled in sample lysis buffer (1× protease inhibitor cocktail, 62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 4% β-mercaptoethanol) for 10 min, then separated on ice to cool, centrifuged at 14,000 g for 5 min. Total protein content in the supernatant was measured by Lowry method. Proteins were boiled for 5 min in sample loading buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 4% β-mercaptoethanol, 0.0005% bромophenol blue). Samples were performed electrophoresis on the Tris-Tricine gel with 70 μg total protein in each lane. After the gel was transferred to PVDF membrane, the membrane was boiled in PBS for 15 min before blocking with a solution of 5% milk in TBS-Tween (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Tween-20) for 1 h at room temperature. Blotting was carried out overnight at 4 °C. Aβ proteins were detected by 6E10 (Biologend, 803001) at 1:1000 dilution. Internal control of β-tubulin was probed by polyclonal antibody against it (Sigma, T6199) at 1:1000 dilution. Goat anti-mouse HRP-labelled IgG was used as a secondary antibody.

**Table 2.** List of primers for amplifying target genes.

| Gene   | Primer name | Primer sequence | Restriction enzyme |
|--------|-------------|-----------------|--------------------|
| daf-16 | daf-16-F    | 5′ AAAAATCGCAAGTGACGCAATTCCCAATGAAAAA 3′ | Pst I |
| daf-16 | daf-16-R    | 5′ CGCGAGTTTGGTAACCCCTGGCCATT 3′ | Hind III |
| snk-1  | snk-1-F     | 5′ AAAAATCGCAAGGGTACCCCTGGCCATT 3′ | Pst I |
| snk-1  | snk-1-R     | 5′ CGCGAGTTTGGTAACCCCTGGCCATT 3′ | Hind III |
| hsf-1  | hsf-1-F     | 5′ AAAGTCCAGGAAAAATTGAGCGAAAAATATATATG 3′ | Pst I |
| hsf-1  | hsf-1-R     | 5′ CGCGAGTTTGGTAACCCCTGGCCATT 3′ | Hind III |
| hsp16.2| hsp16.2-F   | 5′ CGGGGTATCACATTCCAGATTTCCTGCGAGATT 3′ | Kpn I |
| hsp16.2| hsp16.2-R   | 5′ CGCGAGTTTGGTAACCCCTGGCCATT 3′ | Xho I |
| sod-3  | sod-3-F     | 5′ CGGGGTATCACATTCCAGATTTCCTGCGAGATT 3′ | Kpn I |
| sod-3  | sod-3-R     | 5′ CGCGAGTTTGGTAACCCCTGGCCATT 3′ | Xho I |

paralyzed. Each worm was gently touched with a platinum loop to identify as the paralysis, nematodes were considered to be paralyzed if they did not move at all or only moved their heads.
Animals were treated with DXN at dose of 0.39 g/kg and 2.34 g/kg by i.g. for 21 days, mice in positive control group administrated piracetam at dose of 0.5 g/kg by i.g. Amnesia was induced by scopolamine at dose of 3 mg/kg by i.p. 30 min after DXN or piracetam administration. Animals in control group were only received normal saline by i.g.. One day prior to the experiment, animals were trained to swim for 60 sec in the absence of the platform. Mice were performed single trial per day for consecutive 5 days, and escape latency of the time mice taken to another RNAi plates and used for paralysis assay as above.

**Quantification of P**_**sod-3::gfp** _expression via fluorescence microscopy._ Transgenic _C. elegans_ strain CF1553 expressing GFP as a reporter for inducible _sod-3_ expression was used in our study. Age-synchronized L1 stage transgenic _C. elegans_ were treated with or without 15 mg/mL DXN for 72 h, the expression of _sod-3::gfp_ was evaluated through measuring the GFP fluorescence intensity of each whole worm. At least 25 randomly selected worms from each group were observed and photographed by fluorescence microscopy on a glass slide. To quantitatively measure the GFP fluorescence intensity in each whole worm, the Image J software (NIH, Bethesda, MD, USA) was used.

**Quantification of Phsp-16.2::gfp expression via fluorescence microscopy._** In the transgenic strain TJ375, GFP is fused to _hsp-16.2_ promoter for reporting the expression of HSP-16.2. Age-synchronized L1 stage _Phsp-16.2::gfp_ worms were treated with or without 15 mg/mL DXN for 48 h, followed by exposure to 20 mM juglone for 24 h. The expression of _hsp-16.2::gfp_ was evaluated through measuring the fluorescence intensity of the GFP reporter. At least 25 randomly selected worms from each group were measured by fluorescence microscopy on a glass slide. To quantitatively measure the GFP fluorescence intensity in the area anterior of the pharyngeal bulb in individual worms, the Image J software (NIH, Bethesda, MD, USA) was used.

**Morris water maze test.** The Morris water maze test was performed as described by Morris (1981). The apparatus was a circular pool of 120 cm diameter filled with water, and maintained at 22.0 ± 0.5 °C by a calefaction stick. A transparent platform of 11 cm diameter 1 cm below the water surface was placed at a fixed point of one quadrant. 4-6 weeks old Kunming mice were provided by the Experiment Animal Center of Lanzhou University. Animals were treated with DXN at dose of 0.39 g/kg and 2.34 g/kg by i.g. for 21 days, mice in positive control group administrated piracetam at dose of 0.5 g/kg by i.g. Amnesia was induced by scopolamine at dose of 3 mg/kg by i.p. 30 min after DXN or piracetam administration. Animals in control group were only received normal saline by i.g.. One day prior to the experiment, animals were trained to swim for 60 sec in the absence of the platform. Mice were performed single trial per day for consecutive 5 days, and escape latency of the time mice taken to swim to the platform was recorded by an automated tracking system (Ethovision XT software). 24 h after the experiment, a probe trial was performed to test for spatial memory.

All experiments were approved by the Animal Care and Use Committee of Lanzhou University, and the methods were carried out in accordance to the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China.

**Statistical analysis.** Statistical analyses were performed by SPSS 17.0 software. The statistical significances of the results were analyzed by using one-way analysis of variance (ANOVA). Except for paralysis assays, log rank survival test was carried out to compare the significance level among treatments. The P value of 0.05 or less was considered to be significant statistically.

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**Author Contributions**

Dejuan Zhi designed the experiment and analysis the data, wrote the manuscript. Dong Wang prepared Figures and Table 1 except for Figures 3C-E, 4, 5, 6C-D, 8E, 1S-D, 3S. Wengu Yang prepared Figures 3C-E, 6C-D, 8E, 1S-D, 3S. Hongyu Li, Shuqian Zhu, Xin Wang, Ningbo Wang, Juan Dong, Meizhu Wang corrected the manuscript. Ziyun Duan prepared Fig. 5. Hongyu Li put forward guideline opinions. Juan Dong prepared Figure 3S, Na Wang prepared RNAi clones of *hsf-1*, *skn-1*, *daf-16*, *sod-3* and *hsp-16.2*. Dongqing Fei and Zhanxin Zhang prepared compounds 1–15 in Figure 4. All authors reviewed and considered the manuscript.

**Additional Information**

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