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Short report

Withanolide A extends the lifespan in human EGFR-driven cancerous *Caenorhabditis elegans*

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ABSTRACT

The conserved EGFR pathway is linked with multiple cancers in humans including breast, ovarian, and lung carcinoma. Withanolide A, one of the major withanolidal active compounds isolated from the *Withania somnifera*, extends lifespan and ameliorates stress resistance in wild-type *C. elegans* by targeting the Insulin/IGF-1 signaling pathway. Up-regulation of IGF1 can transactivate EGFR which inturn reduces longevity and promotes tumor development in an organism. We examined the effects of Withanolide A on the lifespan of a human EGFR-driven *C. elegans* transgenic model exhibiting the multivulva (Muv) phenotype. The results showed that WA extends the lifespan of both wild human EGFR-driven *C. elegans* model (human wild-type tyrosine kinase) as well as models bearing single (L858R), and double mutations (T790M-L858R). The lifespan extension observed in these transgenic strains was 20.35, 24.21 and 21.27%, respectively. Moreover, the reduced fat levels were noticed in both wild-type N2 worms and transgenic strains. These observations support the heathspan promoting effect of WA as lipid-rich diet has been reported to promote tumor development. In view of the fact that most of the well known FDA approved drugs such as gefitinib fail to inhibit the EGFR-associated cancers because of these mutations, the present findings show the potential of Withanolide A as a foreseen future nutraceutical to improve the average survival of cancer patients.

1. Introduction

Human ageing and age-associated diseases are becoming a major global public health challenge and a financial burden for both developed and developing countries. Although people are living longer i.e. the life expectancy has increased but there is no equivalent improvement in the healthspan. Interestingly, numerous research reports have shown that ageing interventions that lead to an extended lifespan also reduce morbidity in most cases (Fontana et al., 2010). Contrary to the saying: "There is no such thing as ageing, and cancer is not related to it" (Peto et al., 1985), it is now widely accepted that the molecular pathways of ageing and cancer are intertwined (De Magalhães, 2013). In fact, some drugs like metformin have shown both anti-ageing and tumor-inhibition potential (Cabreiro et al., 2013; Castillo-Quan and Blackwell, 2016). Although, the biological links between cancer and ageing has been described in several studies (Pinkston, 2006; De Magalhães, 2013; Carrasco-Garcia et al., 2017), the intricacies of it are still a mystery. *C. elegans* has been widely used by researchers as a model organism for conducting chemical biology experiments associated with ageing. Worms normally do not suffer from cancer as they age, however eliminating the function of the gene *gld-1* produces a worm model of cancer (Francis et al., 1995). Interestingly, in this model it has been shown that *C. elegans* longevity mutations are tumor protective (Pinkston, 2006).

Human cancers, such as breast, ovarian, and lung carcinoma are associated with overexpression of epidermal growth factor receptor (EGFR) and the components of this EGFR pathway are highly conserved between humans and *C. elegans* (Chang and Sternberg, 1999; Hynes and Lane, 2005; Sasaki et al., 2013). Mutations activating *C. elegans* EGFR homolog LET-23 results in a MUltiVulva (Muv) phenotype that can be easily seen under stereoscopic microscope (Sternberg, 2005). *C. elegans* Muv phenotype thus provides great opportunity for understanding the anticancer activities of variety of phytomolecules/chemicals. Following the discovery that lifespan can be manipulated by genes, researchers are enthusiastically looking for the novel herbal compounds that can intervene ageing. Natural compounds are highly sought after due to their immense health benefits by targeting multiple molecules or pathways that affect growth, metabolism, reproduction etc. associated with ageing. Among different types of natural products, phytochemicals have gained significant importance for their health beneficial effects

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and today several plant-based compounds have been identified that extend both lifespan and healthspan of an organism (Argyropoulou et al., 2013; Leonov et al., 2015).

Withania somnifera, a green shrub belonging to the family of solanaceae, contains a wide array of secondary metabolites that has been shown to have anti-cancer, immunimodulatory, anti-convulsant, adatogenic and neurological effects (Mirjalili et al., 2009). However, the pharmacological effect is mainly attributed to its steroidal lactones, called withanoloids (Withanolide A, Withanolide D, withanone, withaferin A). Among numerous bioactive secondary metabolites, Withanolide A (WA), a C28-steroidal lactone present in the methanolic extracts of *W. somnifera,* is one of the most promising withanolides of *W. somnifera*. Akhoon and co-workers evaluated the longevity effects of WA in *C. elegans* using 2 μM, 5 μM, 25 μM, and 50 μM doses of WA (Akhoon et al., 2016). The study showed for the first time that WA extends lifespan of *C. elegans* in a dose-dependent manner. Supplementation of 5 μM WA significantly (≤ 0.0001) extended the mean and median lifespan by 29% and 21% respectively. In view of this motivation, we examined the effects of WA on the lifespan of *C. elegans* transgenic strains viz., JG337, JG324 and JG388.

2. Material and methods

2.1. Plant material

WA was obtained from the Natural Remedies (www.naturalremedy.com), India. Different concentrations of WA were prepared by dissolving it in the dimethyl sulfoxide (DMSO). The concentrated WA was added to the NGM plates seeded with *Escherichia coli* (*E. coli)* OP50 and allowed to dry at room temperature (RT). The worms were then transferred to these prepared NGM plates and DMSO (0.1%) was used as a vehicle control in all the experiments.

2.2. Nematode strains

The nematode strains used in this study were wild-type Bristol N2 and the human EGFR transgenic strains of *C. elegans* viz., JG337: *let-23(sy1); jgIs19* [LET-23N::hEGFR-C, ROL-6d], JG324: *sqt-1(jg52); jgIs6* [LET-23::hEGFR-TK [L858R], ROL-6d], and JG388: *sqt-1(jg52); jgIs25* [LET-23::hEGFR-TK [T790M-L858R], ROL-6d, *myo-2p::mCherry*]. The wild-type Bristol N2 was obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota and EGFR transgenic strains were a kind gift of Prof. Jaegal Shim. All strains except wild-type N2 (cultured at 20 °C) were maintained at 15 °C on the NGM plates seeded with *E. coli* OP50 bacteria and the synchronized populations were obtained using the sodium hypochlorite treatment (Stiernagle, 2006).

2.3. Toxicity assays

To evaluate the acute toxicity of WA, the synchronized L4 worms were transferred to the 24-well plate containing liquid NGM and different concentrations (5, 50, 100, 250, and 500 μM) of WA. DMSO (0.1%) was used as a control. The experiments were repeated thrice and the survival of worms was recorded after 30, 60, 90, 120 min and 24 h, using a touch-provoked movement.

2.4. Lifespan analysis

We performed lifespan experiments on triplicate NGM plates with 50 worms per plate at 20 °C. *E. Coli* OP50 was used as a food source and 5 μM WA was uniformly spread over the food. For progeny growth inhibition, 50 μM of 5-fluorodeoxyuracil was added to the NGM plate. To maintain the effect of WA on the worms, plates were changed every 2–3 days. Kaplan–Meier survival assay in Med Calc software ver. 12.7.7.0 was used to plot the survival of worms. The statistical significance was measured using the log-rank significance test and a *P*-value < 0.05 was considered statistically significant.

2.5. Fat content analyses

Fat content was determined using standard Oil red O staining technique as described (O'Rourke et al., 2009). Experimental details are provided in the supplementary data.

2.6. Real-time qPCR

To quantify the effect of WA on let-23, RNA was harvested using the RNAzol reagent (Invitrogen, USA) and converted to cDNA with cDNA synthesis kit (Invitrogen). Gene expression study was performed with Real-Time PCR System (Applied Biosystems) using SYBER green as a fluorescent probe. The data was normalized to act-1 and further analysed with ΔΔCt relative quantization method.

3. Results and discussion

Among all of the model organisms that are commonly used to access toxicity or anti-ageing effects of genes and or compounds, *C. elegans* stands out because of its short life-cycle, fully sequenced genome and 60–80% human gene counterparts. These features make it a powerful tool for the discovery of novel anti-ageing compounds. The different concentrations of WA were assayed for their toxicity effects in wild-type *C. elegans* prior to conducting lifespan experiments. The dose-dependent acute toxicity was determined by allowing the worms to feed on various concentrations of WA (5, 50, 100, 250 and 500 μM) and examined for 24 h survival (Fig. 1). We observed no deaths in 5–100 μM concentrations of WA however 250 and 500 μM concentrations resulted in 10 and 12% deaths in *C. elegans*, respectively. Such findings show that lower doses of WA can be safely used to carry out other experiments in *C. elegans*.

Next, we analysed the effect of 5 μM WA (optimal dose reported by Akhoon and co-workers) on a human EGFR-driven *C. elegans* model which exhibits the Muv phenotype (Bae et al., 2012). We did not notice any significant reduction in the Muv phenotype of worms by WA treatment however results showed that 5 μM WA administration significantly increases the lifespan of both wild (human wild-type tyrosine kinase (TK) domain (LET-23::hEGFR-TK)) and LET-23 mutants a TK domain with the L858R mutation (LET-23::hEGFR-TK[L858R]), or a TK domain with the T790M-L858R mutations (LET-23::hEGFR-TK[T790M-L858R]). WA supplementation extended the mean lifespan of wild-type N2, JG337, JG324 and JG388 worms by 27.74%, 20.35%, 24.21% and 21.27%, respectively (Fig. 2). It is evident from the Fig. 2 that hEGFR-expressing strains have a shorter lifespan than the wild-type N2 worms however after WA treatment, the worms live longer. WA seems to extend lifespan of hEGFR-expressing strains regardless of EGFR activity, because WA did not reduce multivulva phenotype. Of course multivulva formation is not sole phenotype of EGFR activity, because *C. elegans* let-23/EGFR is expressed in various tissues as well as vulva. Therefore, to confirm whether WA has any impact on the let-23/EGFR, the let-23 expressions in WA treated wild-type N2 worms was analysed using the quantitative real-time PCR. WA failed to change the let-23 expressions in N2 *C. elegans*, suggesting that WA mediated lifespan extension is independent of EGFR activity. Such results encourage the ayurvedic or traditional use of Ashwagandha (WA) for its lifespan extending effects as most of the well known anti-cancer drugs such as gefitinib failed to inhibit the EGFR-associated cancers because of these mutations (Ellison et al., 2013). The mechanism of WA mediated longevity has been