

Adverse Effects from Clenbuterol and Ractopamine on Nematode *Caenorhabditis elegans* and the Underlying Mechanism

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Abstract

In the present study, we used *Caenorhabditis elegans* assay system to investigate *in vivo* toxicity from clenbuterol and ractopamine and the possible underlying mechanism. Both acute and prolonged exposures to clenbuterol or ractopamine decreased brood size and locomotion behavior, and induced intestinal autofluorescence and reactive oxygen species (ROS) production. Although acute exposure to the examined concentrations of clenbuterol or ractopamine did not induce lethality, prolonged exposure to 10 µg/L of clenbuterol and ractopamine reduced lifespan. At relatively high concentrations, ractopamine exhibited more severe toxicity than clenbuterol on nematodes. Overexpression of *sod-2* gene encoding a Mn-SOD to prevent induction of oxidative stress effectively inhibited toxicity from clenbuterol or ractopamine. Besides oxidative stress, we found that clenbuterol might reduce lifespan through influencing insulin/IGF signaling pathway; however, ractopamine might reduce lifespan through affecting both insulin/IGF signaling pathway and TOR signaling pathway. Ractopamine more severely decreased expression levels of *daf-16*, *sgk-1*, *skn-1*, and *aak-2* genes than clenbuterol, and increased expression levels of *daf-2* and *age-1* genes at the examined concentration. Therefore, the *C. elegans* assay system may be useful for assessing the possible toxicity from weight loss agents, and clenbuterol and ractopamine may induce toxicity through different molecular mechanisms.

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Introduction

Illegal or unsuitable use of weight loss agents has gradually become a public health concern [1–2]. Clenbuterol, a typical weight loss agent, is a kind of β_2 -adrenergic agonist, and was illegally used as a feed additive to improve production performance and a carcass composition in many countries [3–4]. With the increased use of internet sales, the internet has made this even banned product to be readily accessible for the aim of weight loss or dieting addition [2]. Ractopamine, a synthetic β_2 -adrenoceptor agonist, is now widely used as a feed additive in the United States to promote a reduction in body fat and to enhance muscle growth in cattle, pigs, or turkeys [5].

Among the used weight loss agents, clenbuterol and ractopamine belong to products that may have health hazards upon accidental or intentional exposure and ingestion [2,6]. The *in vitro* study has indicated that clenbuterol exhibited potential toxicity on structure and function of trypsin, an important digestive enzyme, and stimulated guinea-pig heart rate [4,7]. Following consumption of meat or liver from clenbuterol administrated cattle, intoxication

cases were described [7]. Moreover, at least the toxic effects on cardiovascular systems (such as tachycardia and hypertension) are considered to be of clinical relevance [7–9]. More recently, it was further reported that ractopamine administration might cause the myocardial toxicity in dogs [5].

Several toxicological studies have been performed for clenbuterol. Administration of growth-promoting doses of clenbuterol adversely affected the liver function in female pigs [10]. Dietary administration of clenbuterol decreased androgen receptor (AnR) expression in testicle, glucocorticoid receptor (GR) expression in lymphoid tissues, and β -adrenergic receptor (β -AR) expression in targeted organs of chickens [3]. Clenbuterol caused an impairment of collagen turnover by down-regulating MMP-9 activity [11]. Clenbuterol not only enhanced muscle fiber size but also increased expression of GATA-2 protein in skeletal muscle of rat uterus [12]. The preferential involvement of calpain 2 autolysis was found for clenbuterol-induced skeletal muscle remodelling in rats [13]. Overexpression of calpastatin in skeletal muscle of mice prevented clenbuterol-induced muscle hypertrophy and phenotypic shift

[14]. However, so far no toxicological study on ractopamine has been performed.

Nematode *Caenorhabditis elegans*, an important model animal used in various fields, has invariant and fully described developmental program, well-characterized genome, short and prolific life cycle, and small body size [15–16]. The success of *C. elegans* as a model animal has attracted the increased attention in the fields of both biomedical science and toxicology [16–18]. *C. elegans* has been widely accepted and utilized as an important alternative animal model for toxicity testing [16,19–20]. A number of toxicity studies have been conducted with the aid of both lethal and sub-lethal endpoints for metallic salts [21–31], organic compounds [32–36], drugs [37–40], and engineered nanomaterials [41–50]. *C. elegans* is useful for toxicological studies from whole-animal level down to single cell level [51]. A series of studies have found that toxicity for toxicants in *C. elegans* is similar to that observed in mammals [16,19], implying that the toxicological studies performed in *C. elegans* will closely reflect the effects to be observed in mammalian models for most compounds tested.

In the present study, we first compared the toxicity between clenbuterol and ractopamine with the aid of a series of endpoints in *C. elegans*. Moreover, considering the fact that we still know limited information about toxicological mechanism for the clenbuterol and especially the ractopamine, we examined the underlying mechanism for toxicity from clenbuterol and ractopamine. Our results here will be useful for the further understanding multiple toxicities from clenbuterol and ractopamine and the underlying mechanism.

Results

Comparison of lethality and growth in clenbuterol or ractopamine exposed nematodes

Considering the fact that many toxicants at the low concentrations may have the adverse effects on nematodes after prolonged exposure [18,25,44–49], we performed both the acute exposure and the prolonged exposure for clenbuterol or ractopamine. Concentrations of 0.01–5 mg/L were used for acute exposure to clenbuterol or ractopamine. After acute exposure from young adult stage for 24-hr, both clenbuterol and ractopamine did not induce lethality and alteration of body length in nematodes (Fig. 1). Concentrations of 0.01–10 µg/L were used for prolonged exposure to clenbuterol or ractopamine. After prolonged exposure from L1-larvae to the adult stage, although both clenbuterol and ractopamine still did not induce lethality of nematodes, both clenbuterol and ractopamine at concentrations more than 1 µg/L significantly reduced body length of nematodes (Fig. 1).

Comparison of reproduction and locomotion behavior in clenbuterol or ractopamine exposed nematodes

Reproductive organ and neuron may be important secondary targeted organs for toxicants in nematodes [18,44,48–49,52]. Acute exposure to clenbuterol or ractopamine at concentrations of 0.01–0.1 mg/L and prolonged exposure to 0.01 µg/L of clenbuterol or ractopamine did not significantly alter brood size (Fig. 2). Acute exposure to 0.01 mg/L of clenbuterol or ractopamine did not significantly influence locomotion behavior of nematodes (Fig. 2). In contrast, acute exposure to clenbuterol or ractopamine at concentrations more than 1 mg/L and prolonged exposure to clenbuterol or ractopamine at concentrations more than 0.1 µg/L significantly reduced brood size (Fig. 2). Acute exposure to clenbuterol or ractopamine at concentrations more than 0.1 mg/L and prolonged exposure to clenbuterol or ractopamine at concentrations more than 0.01 µg/L significantly decreased

locomotion behavior of nematodes (Fig. 2). More interestingly, we observed that acute exposure to 1–5 mg/L of ractopamine and prolonged exposure to 0.1–10 µg/L of ractopamine exhibited more severe toxicity on locomotion behavior than clenbuterol in nematodes, although ractopamine at the examined concentrations still showed the similar toxicity on brood size to clenbuterol in nematodes (Fig. 2).

Comparison of intestinal autofluorescence in clenbuterol or ractopamine exposed nematodes

Intestine is the primary targeted organ for toxicants in nematodes [18,41,43,48]. Acute exposure to clenbuterol or ractopamine at concentrations of 0.01–0.1 mg/L and prolonged exposure to clenbuterol or ractopamine at concentrations of 0.01–0.1 µg/L did not induce the significant intestinal autofluorescence compared with control (Fig. 3A). In contrast, acute exposure to 5 mg/L of clenbuterol or ractopamine and prolonged exposure to clenbuterol or ractopamine at concentrations of 1–10 µg/L significantly induced the intestinal autofluorescence compared with control (Fig. 3A). More interestingly, we observed that acute exposure to 5 mg/L of ractopamine and prolonged exposure to 1–10 µg/L of ractopamine induced more pronounced intestinal autofluorescence than clenbuterol in nematodes (Fig. 3A).

Comparison of intestinal reactive oxygen species (ROS) production in clenbuterol or ractopamine exposed nematodes

In *C. elegans*, toxicants usually cause damage on animals by inducing oxidative stress [18,29,53]. We further examined the intestinal ROS production in clenbuterol or ractopamine exposed nematodes. After acute exposure, both clenbuterol and ractopamine at the concentration of 5 mg/L induced the significant intestinal ROS production, and acute exposure to ractopamine at the concentration of 5 mg/L resulted in more severe intestinal ROS production than clenbuterol (Fig. 3B). After prolonged exposure, similarly, both clenbuterol and ractopamine at the concentration of 10 µg/L caused significant intestinal ROS production, and prolonged exposure to ractopamine at the concentration of 10 µg/L induced more severe intestinal ROS production than clenbuterol (Fig. 3B). These data suggest that toxicity from clenbuterol or ractopamine may be closely associated with the induction of oxidative stress in nematodes.

Comparison of lifespan in clenbuterol or ractopamine exposed nematodes

We further investigated the effects of exposure to clenbuterol and ractopamine on lifespan in nematodes. Lifespan is an important endpoint and may reflect the long-term effects of a specific toxicant in nematodes [52,54]. Acute exposure to clenbuterol or ractopamine at the concentration of 5 mg/L did not significantly alter lifespan of nematodes (Figs. 4A and 4B). In contrast, prolonged exposure to clenbuterol or ractopamine at the concentration of 10 µg/L significantly reduced the lifespan of nematodes (Figs. 4C and 4D). Moreover, we found that prolonged exposure to ractopamine at the concentration of 10 µg/L more severely inhibited the lifespan of nematodes than clenbuterol (Figs. 4C and 4D).

Overexpression of SOD-2 prevented the toxicity of clenbuterol or ractopamine on nematodes

In *C. elegans*, *sod-2* and *sod-3* genes encode Mn-SODs, which function in protecting animals from the damage from oxidative stress [45,55]. Previous studies have demonstrated that

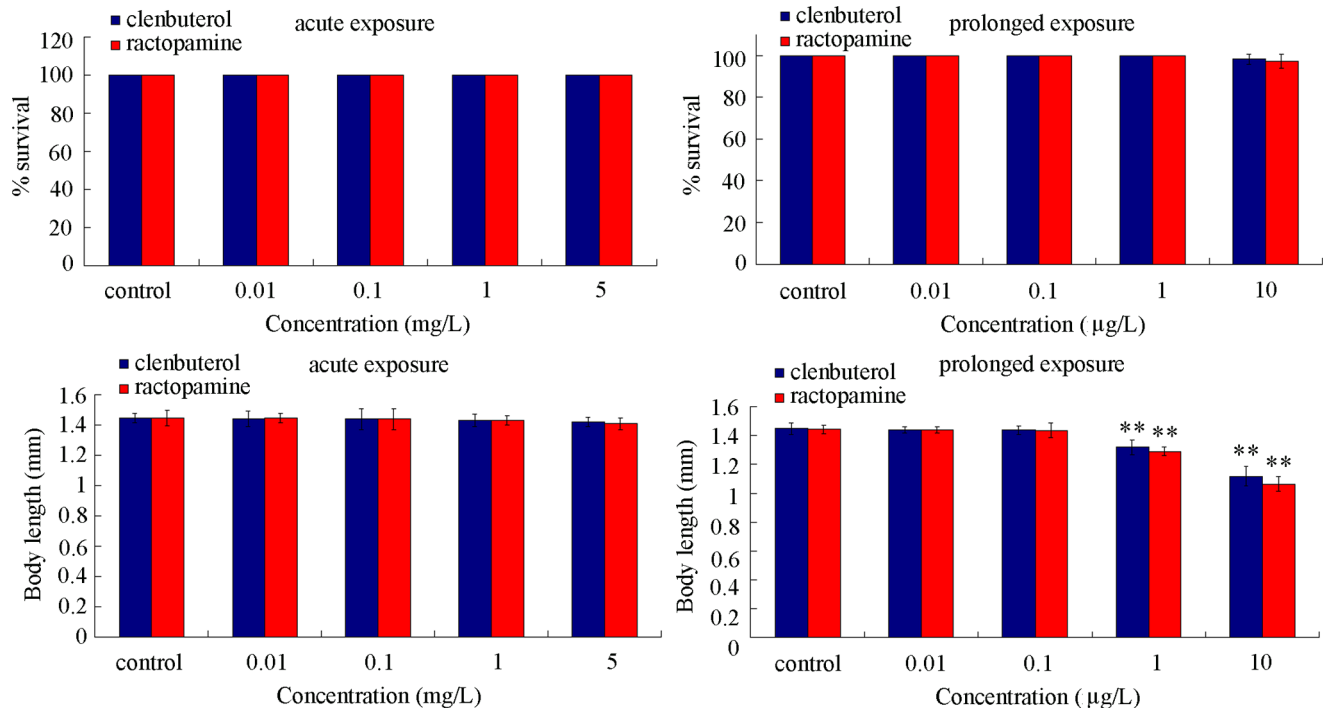


Figure 1. Comparison of lethality and growth in nematodes exposed to different concentrations of clenbuterol or ractopamine. Exposures were performed from the young adult for 24-hr (acute exposure) or from L1-larvae to adult (prolonged exposure). Fifty nematodes were examined per treatment for lethality assay, and twenty nematodes were examined per treatment for growth assay. Bars represent mean \pm S.E.M. $^{**}P < 0.01$.

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overexpression of Mn-SODs inhibited the oxidative stress and prevented the toxicity from metals and nanomaterials [45,55]. To examine the role of oxidative stress in inducing the toxicity from clenbuterol or ractopamine on nematodes, we investigated the effects of overexpression of *sod-2* gene in all cells of nematodes (*Ex(Pdpy-30-sod-2)*) on toxicity from clenbuterol or ractopamine. Transgenic strain of *Ex(Pdpy-30-sod-2)* had no deficits in body length, brood size, head thrash, and body bend, and did not have significant intestinal autofluorescence and intestinal ROS production (Figs. 5A–5E). After prolonged exposure to 10 μ g/L of clenbuterol or ractopamine, we did not detect the significant decreases in body length, brood size, head thrash, and body bend in nematodes overexpressing *sod-2* gene compared with control (Figs. 5A–5C). Moreover, after prolonged exposure to 10 μ g/L of clenbuterol or ractopamine, we did not observe the significant induction of both intestinal autofluorescence and intestinal ROS production in nematodes overexpressing the *sod-2* gene compared with control (Figs. 5D and 5E). Nematodes overexpressing *sod-2* gene had the similar lifespan to that of wild-type N2 (data not shown). After prolonged exposure to 10 μ g/L of clenbuterol or ractopamine, our results further demonstrate that no significant reduction of lifespan was observed in nematodes overexpressing *sod-2* gene compared with control (Fig. 5F).

Molecular mechanism for clenbuterol and ractopamine to reduce the lifespan of nematodes

In *C. elegans*, the aging process is under the control of three major endocrine- and nutrient-sensing signaling pathways, the insulin/insulin-like growth factor (IGF), target of rapamycin (TOR), and germline signaling pathways [56]. To determine the molecular mechanism for clenbuterol or ractopamine to reduce

the lifespan of nematodes, we investigated the expression patterns of genes involved in these three signaling pathways in clenbuterol or ractopamine exposed nematodes. The insulin/IGF signaling pathway includes *daf-2*, *age-1*, *daf-16*, *pdk-1*, *akt-1*, *akt-2*, *sgk-1*, *daf-18*, *prmt-1*, *rle-1*, *smk-1*, *hcf-1*, *hsf-1*, *skn-1*, *akk-2*, and *unc-51* genes (56; Table S1). After prolonged exposure, we found that 10 μ g/L of clenbuterol decreased expression levels of *daf-16*, *sgk-1*, *skn-1*, and *aak-2* genes (Fig. 6A). In contrast, after prolonged exposure, 10 μ g/L of ractopamine not only decreased expression levels of *daf-16*, *sgk-1*, *skn-1*, and *aak-2* genes, but also increased expression levels of *daf-2* and *age-1* genes (Fig. 6A). More interestingly, ractopamine more severely decreased the expression levels of *daf-16*, *sgk-1*, *skn-1*, and *aak-2* genes than clenbuterol (Fig. 6A). TOR signaling pathway includes *daf-15*, *ric-1*, *raga-1*, *rheb-1*, and *pha-4* genes (56; Table S1). After prolonged exposure, clenbuterol did not significantly affect TOR signaling; however, ractopamine increased expression levels of *daf-15* and *ric-1* genes (Fig. 6B). Germline signaling pathway includes *tcer-1*, *kri-1*, *daf-9*, *daf-36*, *daf-12*, *nhr-80*, and *phi-62* genes (56; Table S1). After prolonged exposure, both clenbuterol and ractopamine did not significantly influence the expression levels of genes in germline signaling pathway (Fig. 6C). Therefore, clenbuterol and ractopamine may affect the longevity through different molecular mechanisms in nematodes.

To further confirm the functions of the dysregulated genes in regulating the toxicity formation from clenbuterol or ractopamine, we used the corresponding mutants to investigate the lifespans of these mutants exposed to clenbuterol or ractopamine. With the aid of lifespan as the endpoint, interestingly, we found that the *daf-16(mu86)*, *sgk-1(ok538)*, *skn-1(zu67)*, and *aak-2(ok524)* mutants had the susceptible property to the toxicity of clenbuterol or ractopamine (Fig. 7). In contrast, the *daf-2(e1370)*, *age-1(hx546)*,

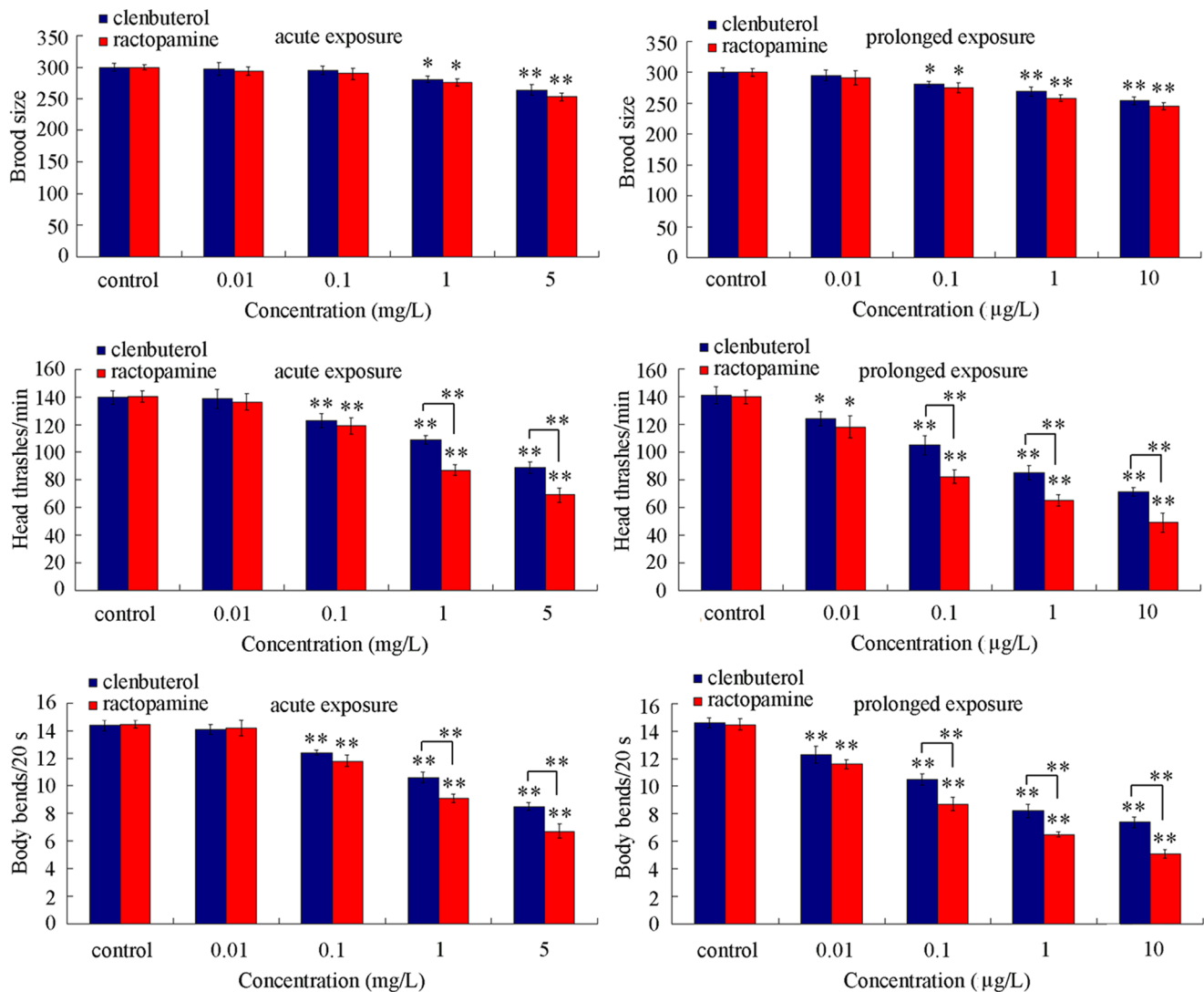


Figure 2. Comparison of brood size and locomotion behavior in nematodes exposed to different concentrations of clenbuterol or ractopamine. Locomotion behavior of nematodes was evaluated by endpoints of head thrash and body bend. Exposures were performed from the young adult for 24-hr (acute exposure) or from L1-larvae to adult (prolonged exposure). Twenty nematodes were examined per treatment for brood size assay, and fifty nematodes were examined per treatment for locomotion behavior assay. Bars represent mean \pm S.E.M. * $P < 0.05$, ** $P < 0.01$. doi:10.1371/journal.pone.0085482.g002

daf-15(m81), and *rict-1(mg360)* mutants had the resistant property to the toxicity of ractopamine (Fig. 7). These data further confirm the involvement of the related signaling pathways in regulating the toxicity formation from clenbuterol or ractopamine.

Discussion

In the present study, we first provide a series of evidence to indicate the usefulness of *C. elegans* assay system in assessing the *in vivo* toxicity of weight loss agents, such as clenbuterol or ractopamine. With the aid of brood size, head thrash, body bend, intestinal autofluorescence, and intestinal ROS production as the endpoints, we detected the toxicity from acute exposure to clenbuterol or ractopamine (Figs. 2 and 3). With the aid of body length, brood size, head thrash, body bend, intestinal autofluorescence, intestinal ROS production, and lifespan as the endpoints, we further detected the toxicity from prolonged exposure to clenbuterol or ractopamine (Figs. 1–4). Using head thrash and body bend as the endpoints, we could observe the potential

toxicity from acute exposure to 0.1 mg/L of clenbuterol or ractopamine, and the potential toxicity from prolonged exposure to 0.01 µg/L of clenbuterol or ractopamine (Fig. 2). Therefore, *C. elegans* may be a relatively sensitive assay system for toxicity assessment of food additives. Especially, the prolonged exposure assay system may be more suitable for detecting the potential toxicity of long-term adverse effects of weight loss agents. For example, prolonged exposure to 1–10 µg/L of clenbuterol or ractopamine significantly decreased the body length of nematodes (Fig. 1). In contrast, acute exposure to all the examined concentrations of clenbuterol or ractopamine did not noticeably influence the body length of nematodes (Fig. 1). Nevertheless, we did not observe the induction of lethality in nematodes exposed to clenbuterol or ractopamine at the examined concentrations (Fig. 1). The careful selection of suitable and sensitive endpoints for toxicity assessment of weight loss agents should be paid attention to, since the toxicity of illegal weight loss agents may be not so severe as heavy metals [21,23,25–30] or organic pesticides [33–34].

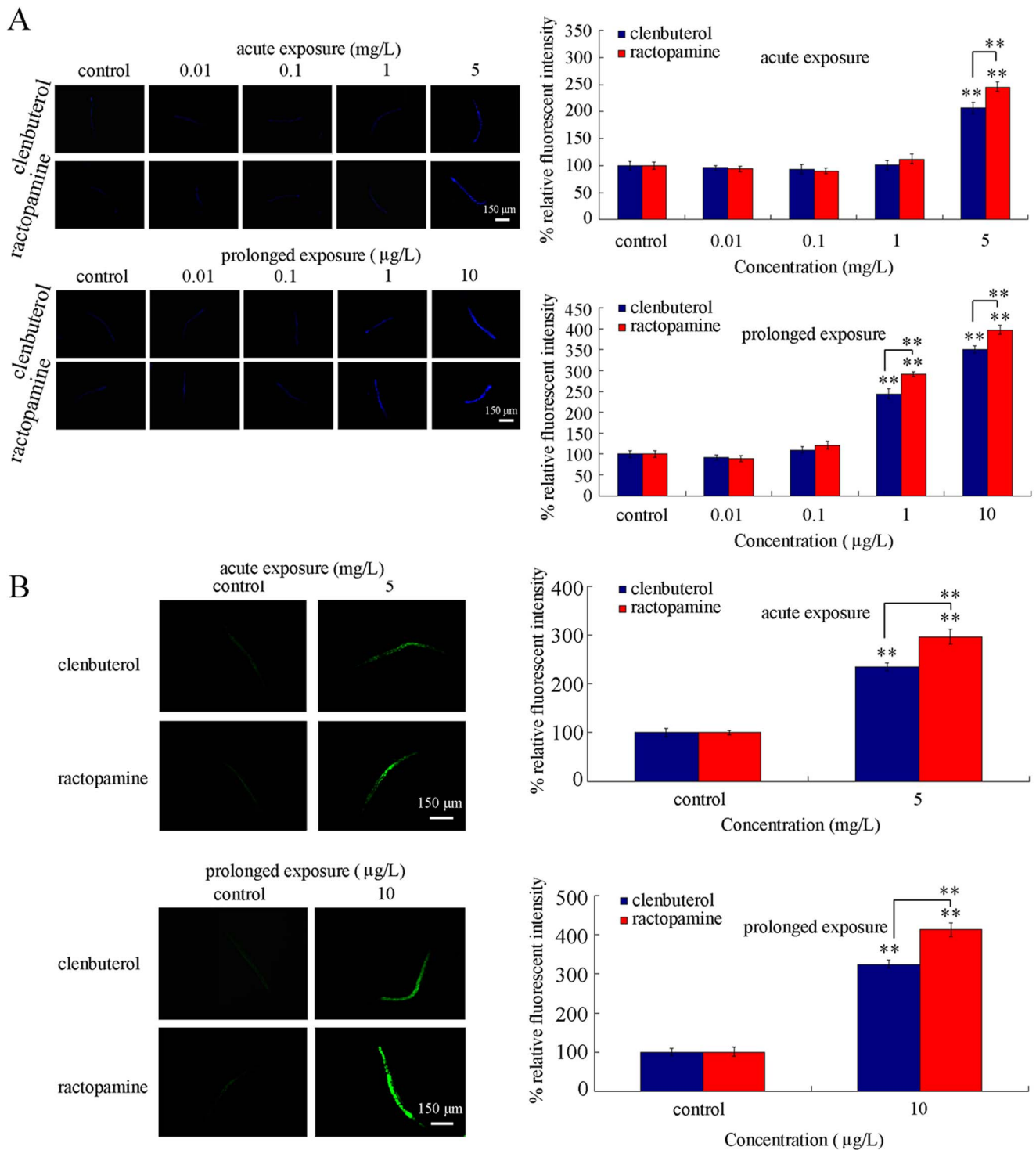


Figure 3. Comparison of intestinal autofluorescence (A) and ROS production (B) in nematodes exposed to clenbuterol or ractopamine. Exposures were performed from the young adult for 24-hr (acute exposure) or from L1-larvae to adult (prolonged exposure). Twenty nematodes were examined per treatment. Bars represent mean \pm S.E.M. ****** P <0.01. doi:10.1371/journal.pone.0085482.g003

Based on our data, we indicate that ractopamine may somewhat have more severe toxicity than clenbuterol in nematodes. With the aid of head thrash, body bend, intestinal autofluorescence, and intestinal ROS production as the endpoints, after acute exposure, we detected the toxicity differences between clenbuterol and

ractopamine at concentrations of 1 and/or 5 mg/L (Figs. 2 and 3). With the aid of head thrash, body bend, intestinal autofluorescence, intestinal ROS production, and lifespan as the endpoints, after prolonged exposure, we observed the toxicity differences between clenbuterol and ractopamine at relatively high concentrations

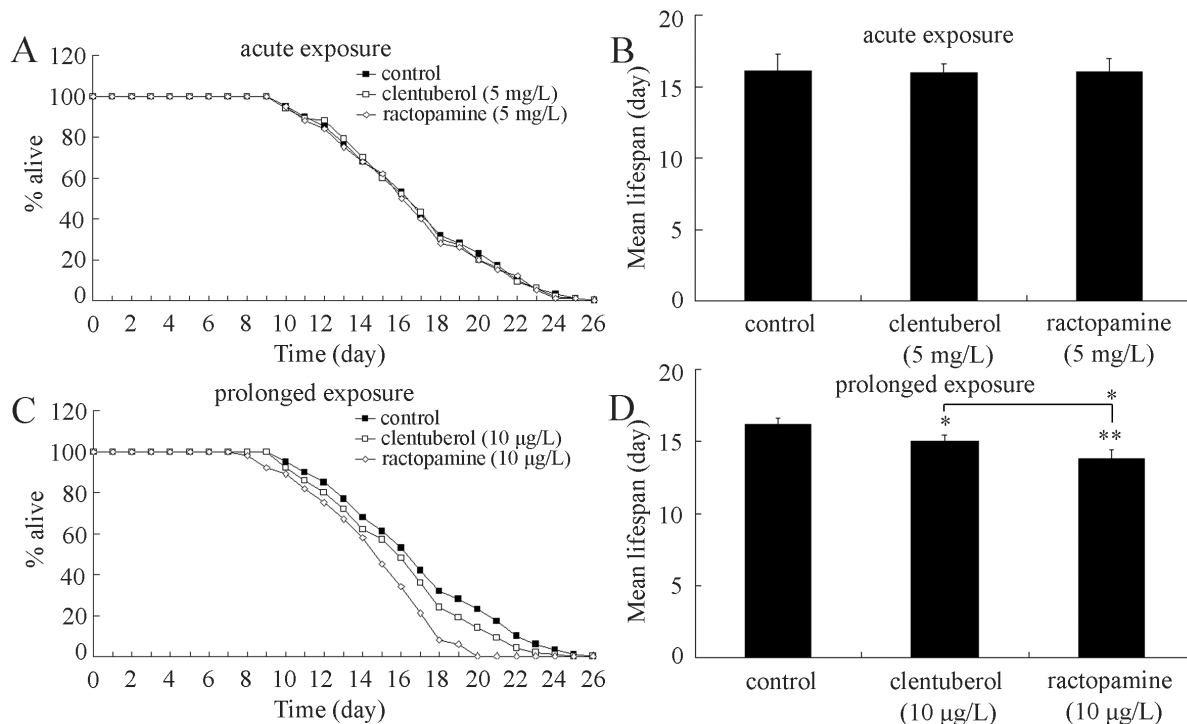


Figure 4. Comparison of lifespan in nematodes exposed to clenbuterol or ractopamine. (A and C) Lifespan curves of nematodes exposed to clenbuterol or ractopamine. (B and D) Comparison of mean lifespans in nematodes exposed to clenbuterol or ractopamine. Exposures were performed from the young adult for 24-hr (acute exposure) or from L1-larvae to adult (prolonged exposure). Thirty nematodes were examined per treatment. Bars represent mean \pm S.E.M. * $P < 0.05$, ** $P < 0.01$. doi:10.1371/journal.pone.0085482.g004

(Figs. 2–4). Considering the fact that the toxicity for toxicants in *C. elegans* may be similar to that observed in mammals [16,19], our data here imply the possible potential more severe toxicity of ractopamine than clenbuterol in mammals.

Oxidative stress is a key mechanism for toxicants to induce the damage on nematodes [16,18]. Our data demonstrate that both clenbuterol and ractopamine induced the significant intestinal ROS production (Fig. 3B). Moreover, with the aid of the transgenic strain of *Ex(Pdpy-30-sod-2)*, we found that overexpression of *sod-2* gene in all cells effectively prevented the potential toxicity from clenbuterol or ractopamine on growth, reproduction, locomotion behavior, intestinal development and lifespan in nematodes (Fig. 5). Therefore, oxidative stress is an important mechanism for clenbuterol and ractopamine to induce the damage on nematodes.

Moreover, we found that clenbuterol and ractopamine also induce the toxicity on nematodes through other mechanisms. In *C. elegans*, our data demonstrate that clenbuterol and ractopamine caused the reduction in lifespan of animals possibly through different molecular mechanisms. Clenbuterol might reduce the lifespan of nematodes through influencing the insulin/IGF signaling pathway; however, ractopamine might reduce the lifespan of nematodes through affecting both the insulin/IGF signaling pathway and the TOR signaling pathway (Fig. 6). In *C. elegans*, insulin/IGF-1 receptor (DAF-2) activates its tyrosine kinase activity and initiates a cascade of phosphorylation events that activate several kinases: phosphatidylinositol 3-kinase (PI3K/AGE-1), 3-phosphoinositide-dependent kinase 1 (PDK-1), and serine/threonine-protein kinase (SGK-1) [56]. Ultimately, SGK-1 phosphorylates and inactivates the FOXO transcription factor DAF-16 and thereby blocks the transcription of targeted genes

[57]. The catalytic subunit of AMP-activated protein kinase, AAK-2, is necessary for the long lifespan of *daf-2* mutants, which explains the energy mechanism [58]. TOR exists in two complexes, TORC1 and TORC2, and TORC1 and TORC2 contain different coactivators, DAF-15/Raptor and RICT-1/Rictor [59]. We hypothesize here that the more reduction in lifespan in ractopamine exposed nematodes than in clenbuterol exposed nematodes may at least due to the induction of more severe alteration in genes required for aging control and the changes of more signaling pathways. Our data suggest that ractopamine more severely decreased the expression levels of *daf-16*, *sgk-1*, *skn-1*, and *aak-2* genes than clenbuterol (Fig. 6A). In addition, ractopamine not only decreased the expression levels of *daf-16*, *sgk-1*, *skn-1*, and *aak-2* genes like clenbuterol, but also increased the expression levels of *daf-2* and *age-1* genes (Fig. 6A).

Previous studies further imply the possible crosstalk between insulin/IGF signaling pathway and oxidative stress in clenbuterol or ractopamine exposed nematodes. The transcription factor Skinhead (SKN-1) regulates resistance to oxidative stress and expression of detoxification genes in response to reduced insulin/IGF-1 signaling [60]. That is, the activated insulin/IGF signaling may at least partially contribute to the induction or regulation of oxidative stress in clenbuterol or ractopamine exposed nematodes.

In conclusion, we provide the evidence in the present study to demonstrate that the *C. elegans* assay system was useful for assessment of possible *in vivo* toxicity from weight loss agents such as clenbuterol and ractopamine. Our data imply that ractopamine exposure might induce more severe toxicity than clenbuterol exposure in nematodes. Besides the oxidative stress, our results demonstrate that both insulin/IGF-1 signaling pathway and TOR signaling pathway were involved in the regulation of toxicity

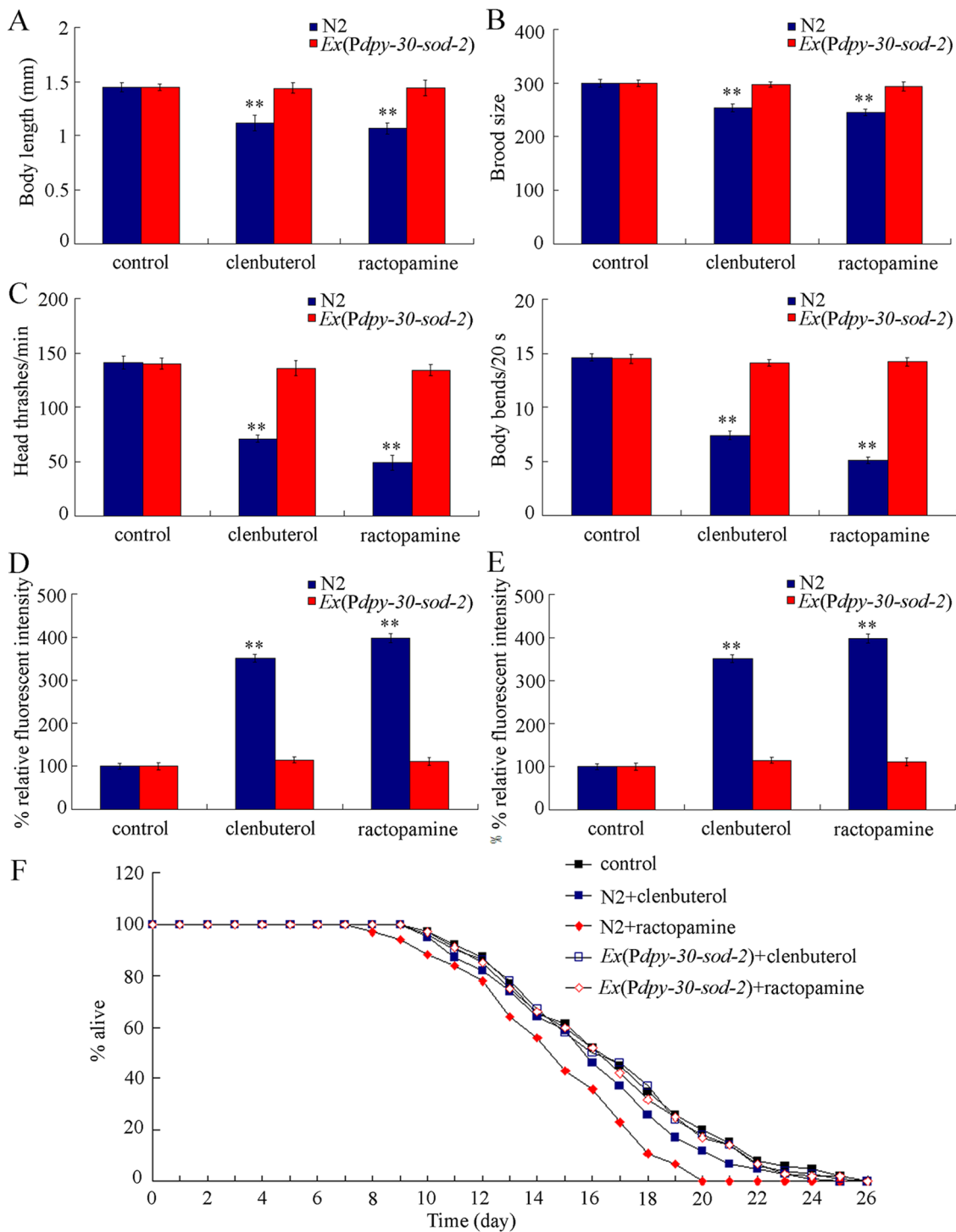


Figure 5. Effects of overexpression of *sod-2* gene on toxicity from clenbuterol or ractopamine in *C. elegans*. (A) Effects of overexpression of *sod-2* gene on growth in clenbuterol or ractopamine exposed nematodes. Twenty nematodes were examined per treatment. (B) Effects of overexpression of *sod-2* gene on brood size in clenbuterol or ractopamine exposed nematodes. Twenty nematodes were examined per treatment. (C) Effects of overexpression of *sod-2* gene on locomotion behavior in clenbuterol or ractopamine exposed nematodes. Fifty nematodes were examined per treatment. (D) Effects of overexpression of *sod-2* gene on intestinal autofluorescence in clenbuterol or ractopamine exposed nematodes. Twenty nematodes were examined per treatment. (E) Effects of overexpression of *sod-2* gene on intestinal ROS production in clenbuterol or ractopamine exposed nematodes. Twenty nematodes were examined per treatment. (F) Effects of overexpression of *sod-2* gene on lifespan in clenbuterol or ractopamine exposed nematodes. Thirty nematodes were examined per treatment. Exposures were performed from L1-larvae to adult (prolonged exposure) at the concentration of 10 $\mu\text{g/L}$. Bars represent mean \pm S.E.M. ** $P < 0.01$. doi:10.1371/journal.pone.0085482.g005

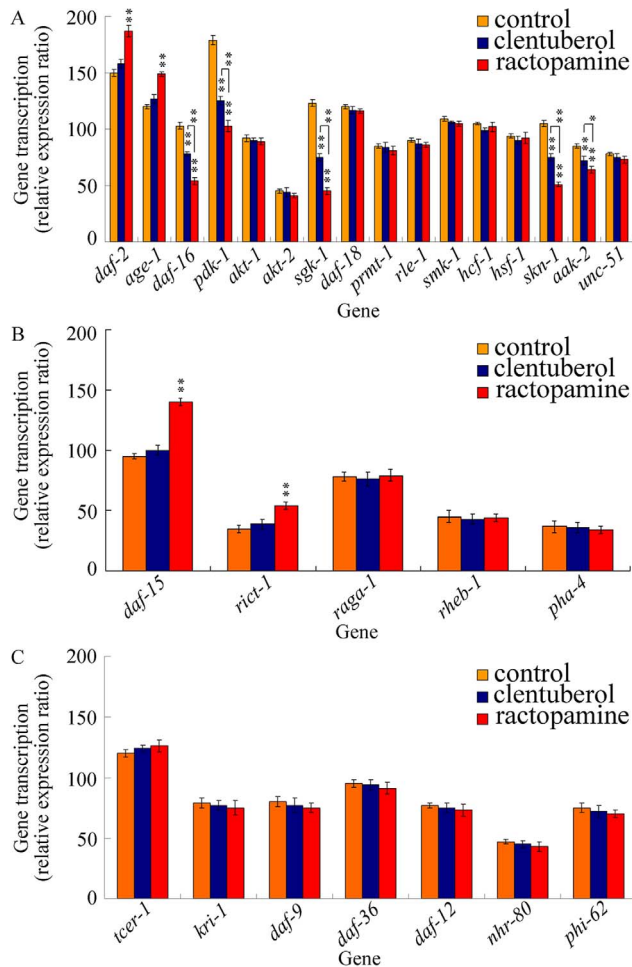


Figure 6. Effects of clenbuterol or ractopamine exposure on expression patterns of genes required for aging control. (A) Effects of clenbuterol or ractopamine exposure on expression patterns of genes in insulin/IGF-1 signaling pathway. (B) Effects of clenbuterol or ractopamine exposure on expression patterns of genes in TOR signaling pathway. (C) Effects of clenbuterol or ractopamine exposure on expression patterns of genes in germline signaling pathway. Exposures were performed from L1-larvae to adult (prolonged exposure) at the concentration of 10 $\mu\text{g/L}$. Bars represent mean \pm S.E.M. ****** $p < 0.01$. doi:10.1371/journal.pone.0085482.g006

induction from clenbuterol or ractopamine in nematodes. Moreover, we hypothesize that clenbuterol and ractopamine might induce the toxicity on nematodes through different molecular mechanisms. Our data here will be helpful for our further understanding the potential damage from illegal use of weight loss agents on health of human and animals and the future design of effective strategies against the adverse effects from weight loss agents.

Materials and Methods

Reagents and strain preparation

Clenbuterol and ractopamine were obtained from Sigma-Aldrich (St. Louis, MO, USA), and their purities were 95% and 99.5%, respectively. Exposure concentrations for clenbuterol or ractopamine were 0.01–5 mg/L (acute exposure) or 0.01–10 $\mu\text{g/L}$ (prolonged exposure).

Nematodes used were wild-type N2, mutants of *daf-2(31370)*, *age-1(hx546)*, *daf-16(mu86)*, *pdk-1(mg142)*, *sgk-1(ok538)*, *skn-1(zu67)*, *aak-2(ok524)*, *daf-15(m81)*, and *rict-1(mg360)*, and transgenic strain of *Ex(Pdpy-30-sod-2)*, which over-expresses the *sod-2* gene in all cells. Nematodes were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 at 20°C [15]. Gravid nematodes were washed off the plates into centrifuge tubes, and were lysed with a bleaching mixture (0.45 M NaOH, 2% HOCl). Age synchronous populations of L1-larvae or young adult nematodes were obtained by the collection [21]. Nematodes were washed with a modified K medium (50 mM NaCl, 30 mM KCl, 10 mM NaOAc, pH 5.5) [61]. Exposures were performed from young adult for 24-hr (acute exposure) or from L1-larvae to adult (prolonged exposure) in K medium of 12-well sterile tissue culture plates at 20°C incubator in the presence of food.

Lethality and growth

Lethality was evaluated by the percentage of survival animals. Following exposure, inactive ones were scored under a dissecting microscopy and nematodes were judged to be dead if they did not respond to stimulus using a small, metal wire. Fifty nematodes were examined per treatment. Growth was assessed by the body length, which was determined by measuring the flat surface area of nematodes using the Image-Pro® Express software. Twenty nematodes were examined per treatment. Three replicates were performed.

Brood size and locomotion behavior

Reproduction was assessed by the brood size of adult nematodes. To assess brood size, we counted the number of offspring at all stages. Nematodes were transferred daily to new agar plates, until the completion of the egg laying period. Hatched progeny were allowed to grow to L1/L2 stage and counted manually. Twenty nematodes were examined per treatment. Three replicates were performed.

Locomotion behaviors of nematodes were evaluated by head thrash and body bend [62]. To assay head thrash, every examined nematode was transferred into a microtiter well containing 60 μL of modified K medium on the top of agar, and head thrashes were counted for 1-min after a 1-min recovery period. A thrash was defined as a change in the direction of bending at the mid body. To assay body bend, nematodes were picked onto a second plate and scored for the number of body bends in an interval of 20 sec. A body bend was counted as a change in the direction of the part of the nematodes corresponding to the posterior bulb of the pharynx along the y axis, assuming that the nematode was traveling along the x axis. Fifty nematodes were examined per treatment. Three replicates were performed.

Intestinal autofluorescence

Intestinal autofluorescence caused by lysosomal deposits of lipofuscin can accumulate over time in aging nematodes [63–64]. Images were collected for endogenous intestinal fluorescence using a 525-nm bandpass filter and without automatic gain control in order to preserve the relative intensity of different animal's fluorescence. Observations of fluorescence were recorded and color images subjected to a common exposure time were taken for the documentation of results with Magnafire® software (Olympus, Irving, TX, USA). Lipofuscin levels were measured using ImageJ Software (NIH Image) by determining average pixel intensity in each animal's intestine. Twenty nematodes were examined per treatment. Three replicates were performed.

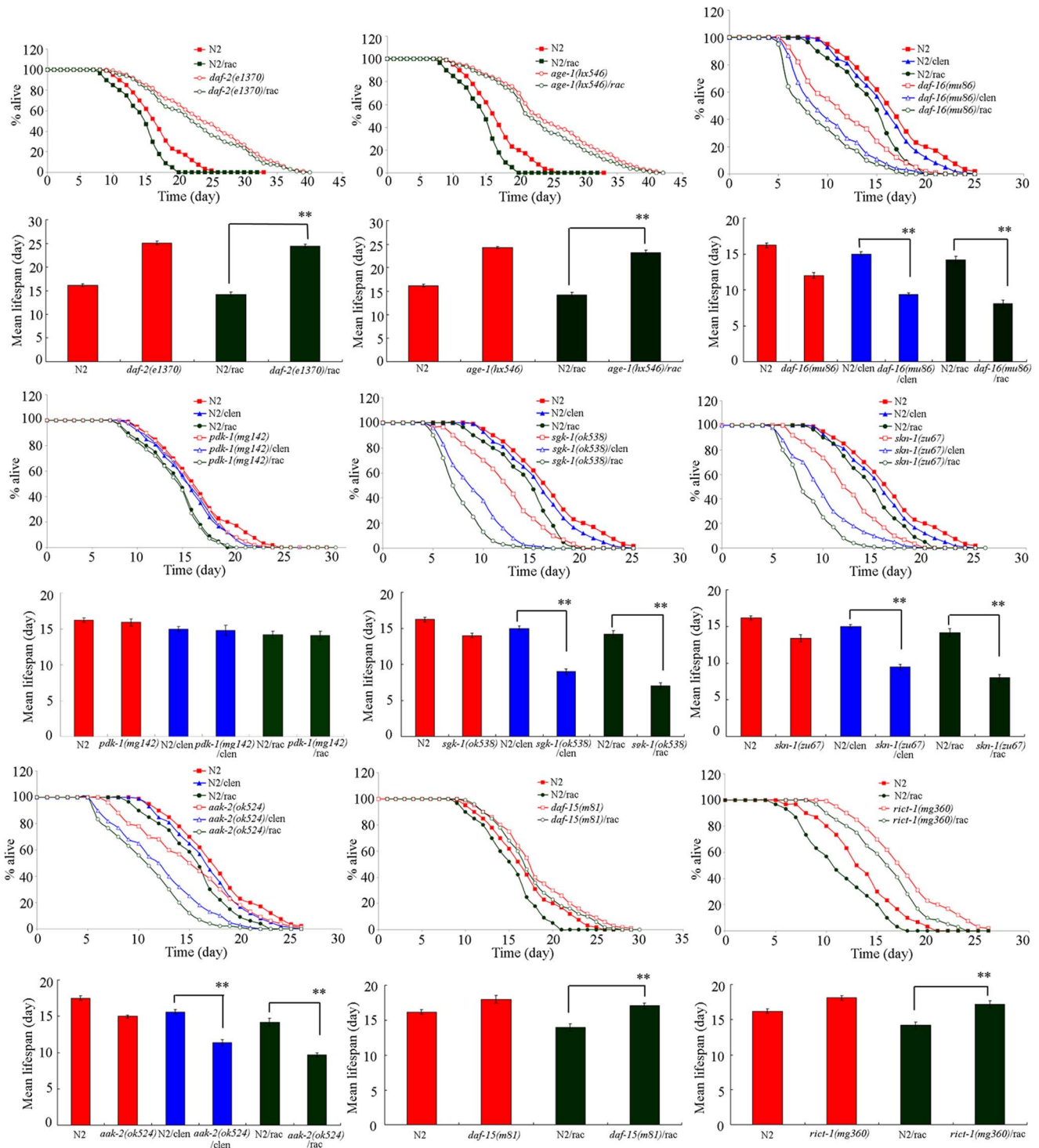


Figure 7. Lifespans in wild-type and mutants exposed to clenbuterol or ractopamine. Exposures were performed from L1-larvae to adult (prolonged exposure) at the concentration of 10 μ g/L. Thirty nematodes were examined per treatment. clen, clenbuterol; rac, ractopamine. Bars represent mean \pm S.E.M. ** $P < 0.01$. doi:10.1371/journal.pone.0085482.g007

ROS production

To quantify whether the clenbuterol or ractopamine exposure activated the oxidative damage, ROS production was assayed. The examined nematodes were transferred to M9 buffer containing 1 μ M of 5-(and-6)-chloromethyl-2',

7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) to pre-incubate for 3-h at 20°C, and then mounted on agar pads for examination with a laser scanning confocal microscope (Leica, TCS SP2, Bensheim, Germany) at 488 nm of excitation wavelength and 510 nm of emission filter. Relative fluorescence intensities of the intestines were semi-quantified as

described previously [30]. The semiquantified ROS was expressed as relative fluorescent units (RFU). Twenty nematodes were examined per treatment. Three replicates were performed.

Lifespan assay

Lifespan assay was performed basically as described [65–66]. In the test, the hermaphrodites were transferred daily for the first 4 days of adulthood. Nematodes were checked every day and would be scored as dead when they did not move even after repeated taps with a pick. Thirty nematodes were examined per treatment. For lifespan, graphs are representative of at least three trials.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted using RNeasy Mini Kit (Qiagen). Total nematode RNA (~1 µg) was reverse-transcribed using a cDNA Synthesis kit (Bio-Rad Laboratories). Quantitative reverse transcription PCR (RT-PCR) was run at the optimized annealing temperature of 58°C. Relative quantification of the targeted genes in comparison to the reference *act-1* gene was determined, and the final results were expressed as the relative expression ratio (between targeted genes and reference gene). The designed primers for targeted genes and reference *act-1* gene were shown in Table S2.

DNA construct and germline transformation

To construct plasmid of *Pdpy-30-sod-2*, *dpy-30* gene promoter fragment (1907 bp, PstI/BamHI) was subcloned into the pPD95_75 vector, and the full length of *sod-2* cDNA was inserted into the site of SmaI/KpnI of the pPD95_75 vector behind *Pdpy-*

30 fragment. Transgenic nematodes of *Ex(Pdpy-30-sod-2)* were generated as described [67]. Plasmids were injected as a mix at 20 ng/µL using *Pdop-1::rffp* as a transgenic marker.

Statistical analysis

All data were expressed as means ± standard error of the mean (S.E.M.). Statistical analysis was performed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) followed by Tukey post-hoc test was used to determine the significance of differences between the groups. Probability levels of 0.05 and 0.01 were considered statistically significant. The lifespan data were statistically analyzed using a 2-tailed 2 sample *t*-test (Minitab Ltd., Coventry, UK).

Supporting Information

Table S1 Information for genes required for aging control in *C. elegans*.

(DOC)

Table S2 Primers used for quantitative real-time polymerase chain reaction (PCR).

(DOC)

Author Contributions

Conceived and designed the experiments: WG DW. Performed the experiments: ZZ QW YZ ML HL. Analyzed the data: LS. Contributed reagents/materials/analysis tools: ZZ WG. Wrote the paper: DW.

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