



Short communication

## Subcutaneous daidzein administration enhances recovery of skilled ladder rung walking performance following stroke in rats



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### HIGHLIGHTS

- We utilized the skilled ladder rung walking task as a behavioral measure.
- We tested whether subcutaneous daidzein administration enhanced post-stroke recovery.
- Treatment began at the time of stroke.
- Rats treated with daidzein recovered better than rats treated with vehicle alone.

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### ABSTRACT

Stroke is a devastating event which can result in permanent disability. Due to the lack of treatments available for use after stroke, compounds which work to limit cell loss, reduce behavioral deficits, and enhance recovery of function are needed. The isoflavone daidzein has been demonstrated to be neuroprotective when fed to rats beginning prior to stroke. Herein, we tested whether subcutaneous delivery of daidzein beginning at the time of stroke reduced injury and/or enhanced functional recovery over 14 days after stroke. Baseline performance on the skilled ladder rung walking task was recorded immediately before stroke (Day 0). Rats then underwent a unilateral permanent middle cerebral artery occlusion and received a subcutaneous minipump containing either daidzein dissolved in vehicle or vehicle alone. Performance on the skilled ladder rung walking task was recorded again on Day +3, Day +7, and Day +14 post-stroke. Rats were then euthanized and brains were collected for lesion volume analysis. The numbers of slight and deep forelimb slips on the task were recorded for 3 trials for each rat per day. Rats treated with daidzein exhibited fewer deep slips over the course of the experiment than rats which received only vehicle ( $p < 0.05$ ). No difference was detected in total forelimb slips or slight slips ( $p > 0.05$ ). Lesion volume was not different between groups ( $p > 0.05$ ). No differences were found in weight between groups during the study ( $p > 0.05$ ).

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An ischemic stroke frequently produces lasting damage to brain tissue, which often results in a long-term reduction in motor, sensory, or cognitive function. These deficits, alone or in combination, can significantly reduce quality of life measures [1]. On average, one person in the United States suffers from a stroke every 40 s, with approximately 795,000 new or recurrent strokes occurring each year [1]. Currently, no approved treatments are available to enhance recovery of function after stroke injury, and the

identification of potentially useful compounds to aid in enhancing recovery of function after stroke has been identified by many as a priority [2].

Natural product-based interventions may represent effective ways to reduce the risk and initial injury of stroke in human populations [3]. Specifically, soy-based diets have been identified by our lab and others as being particularly effective in reducing stroke injury in adult male and female rats when provided before the onset of stroke [4–6]. There is evidence that soy diet-mediated neuroprotection and recovery of function may be at least partially mediated by the isoflavones daidzein and/or genistein, which are the two main isoflavones found in soy-based diets [5,7–10]. Both daidzein and genistein can cross the blood brain barrier [11,12].

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Since stroke is a sudden event, preventative treatments such as dietary intervention are not always possible, even if patient risk factors have been identified. For this reason, the discovery of neuroprotective or restorative compounds for use at or after the time of stroke is a critical need. For this reason, in the current study we examined whether the soy isoflavone daidzein may be useful in reducing stroke severity, reducing lesion volume, and/or enhancing functional recovery after stroke when administered via subcutaneous infusion beginning at the time of stroke.

To accomplish this, the skilled ladder rung walking task was used to gauge motor function in the contralesional forelimb before and after stroke, using previously described methods [13]. Prior to assignment to groups, all twenty four rats in the study were individually exposed to a skilled ladder rung walking apparatus and allowed to cross the apparatus three consecutive times each for two days in order to become familiar with the task. At the beginning of each trial, rats were placed on a platform at one end of a horizontal ladder obstacle with randomly spaced rungs and were filmed with a high definition video camcorder (Canon Vixia HF21) while walking across the bars to a platform at the opposite end. Three complete trials were recorded for each testing day.

On Day 0, baseline performance on the skilled ladder rung walking task was recorded to video as above. Each rat then received a permanent unilateral middle cerebral artery occlusion (MCAO) surgery to induce a stroke in the left hemisphere of the brain and received a subcutaneous osmotic minipump containing either daidzein plus vehicle or vehicle alone, as described below. Skilled ladder rung walk performance was assessed after MCAO on Day +3, Day +7, and Day +14 to assess behavioral deficits and gauge recovery of function in each group. In addition, each rat's mass was measured and recorded on each testing day.

Following the conclusion of the study, all of the skilled ladder rung walking apparatus videos were viewed and scored by an observer who was blind with respect to treatment group. Performance by the contralesional, impaired forelimb was assessed and errors on the task were scored as either slight or deep slips using criteria reported previously [13], which were noted separately. At the conclusion of analyses, the numbers of total, slight, and deep slips were compared between groups over time using a Two-way repeated measures ANOVA (Prism 6.0).  $p$ -values less than 0.05 were considered significant.

After baseline skilled ladder rung walking performance was recorded, rats were randomly assigned to either the vehicle only control group or the daidzein plus vehicle treatment group. Twenty-four (24) adult male Long Evans Hooded rats ( $N=12$ /treatment group) underwent a permanent unilateral middle cerebral artery occlusion (MCAO) in order to produce a stroke. The surgery was performed as published previously [4]. Briefly, rats were anesthetized with isoflurane (5% in oxygen) until unresponsive. Rats were placed on a heated water circulator and secured in a stereotaxic device that allows for continuous anesthesia and access to the dorsal and ventral aspects of the rat as needed. Rats were maintained on isoflurane (1–2.5% in oxygen) during surgery. Upon reaching a surgical plane of anesthesia, the left lateral aspect of the skull was exposed and a craniotomy was performed to expose the middle cerebral artery (MCA) at the point where it exits the rhinal fissure. The MCA was then ligated and transected. Rats then underwent a permanent left common carotid artery (CCA) occlusion and a temporary (15 min) occlusion of the contralateral CCA. Immediately following MCAO, an Alzet osmotic minipump (Model 2002) was implanted beneath the skin via a mid-scapular incision. Each pump contained sterile 0.9% saline with either 12 mM daidzein in dimethylsulfoxide (DMSO) for the daidzein treatment group ( $N=12$ ) or DMSO alone for the vehicle control group ( $N=12$ ). Rats were then removed from anesthesia and allowed to recover before being returned to their home cages. All surgical and animal

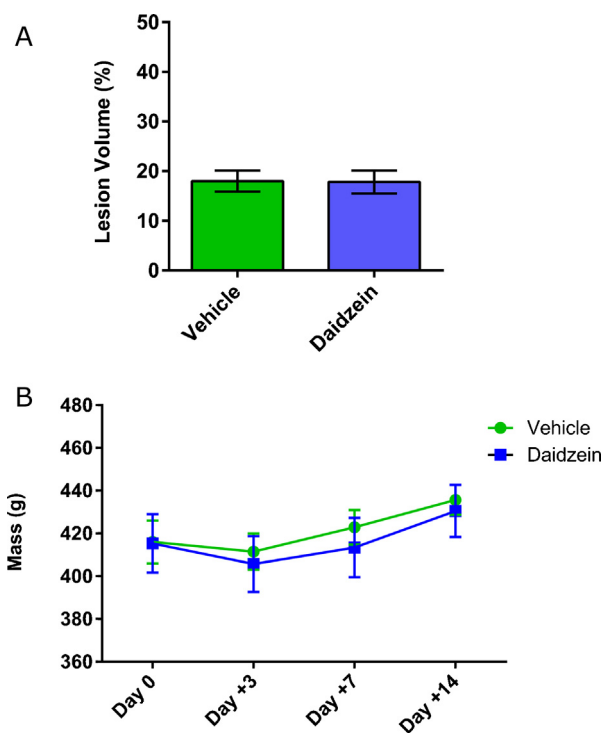
care procedures were approved by the Southern Illinois University Carbondale Institutional Animal Care and Use Committee.

The concentration of daidzein for delivery via a subcutaneous route was chosen based on findings published previously by others [12,14] and was adapted to fit our protocol. For rats in the daidzein treatment group, osmotic minipumps were filled with a 12 mM daidzein solution containing DMSO and 0.9% saline (1:1). After subcutaneous placement, the minipumps delivered daidzein at a rate of 0.044 mg daidzein/12  $\mu$ l solution/day for 14 days, resulting in a dose of 0.10 mg daidzein/kg/day for a 420 g rat. Rats in the vehicle only group received pumps filled with only DMSO/saline solution and not daidzein.

On Day +14, all rats were euthanized by an overdose of sodium pentobarbital (100 mg/kg i.p.) and perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were removed, post-fixed in 4% paraformaldehyde for 24 h at 4 °C, cryoprotected by sinking in 30% sucrose, and then flash frozen. Brains were stored at –80 °C until sectioning. Frozen brains were sectioned cut on a cryostat at 50  $\mu$ m, and alternate series were mounted and stained with cresyl violet (CV) for lesion volume calculations and cytoarchitectural analysis. CV-stained brain sections were digitized using a Nikon Super Coolscan 5000 ED fitted with a medical slide adapter. Image J software [15] was then used to measure and quantify the lesion volume, as performed previously [4]. For this, the total areas of both the injured and intact hemispheres were measured separately on every sixth section throughout the rostrocaudal extent of the lesion, beginning with the most rostral section. The area of undamaged tissue in the lesioned hemisphere was divided by the area of the controlled, non-injured contralateral cortex to determine the percent difference in tissue volume between hemispheres in each individual section. This value was then subtracted from 100% to calculate the percent area of the lesion and then averaged to determine a mean percent difference across the rostrocaudal extent of the lesion. Next, the rostrocaudal length of the lesion within each brain was determined and expressed as a percentage of a maximum of 14 mm, which was chosen to represent the approximate mean rostrocaudal length of the rat cerebral cortex, based on our calculations (not shown). Finally, the percentage of the lesion length of each brain was then multiplied by the mean lesion area calculated in previous steps to determine the total volume of each brain lesion as a percentage of the total hemispheric volume. The resulting lesion volumes were compared using a  $t$ -test (Prism 6.0; Graphpad Software), in which  $p < 0.05$  was considered significant (Fig. 1). All procedures and analyses were performed in a random, blinded fashion, including MCAO and minipump surgeries.

Baseline performance (Day 0) was not significantly different between treatment groups for total slips ( $p > 0.05$ ; Fig. 2), slight slips ( $p > 0.05$ ; data not shown), or deep slips ( $p > 0.05$ ; data not shown). No differences were detected after stroke in total forelimb slips ( $p > 0.05$ ; Fig. 2) or slight forelimb slips ( $p > 0.05$ ; data not shown) between groups. However, rats in the daidzein treatment group exhibited fewer deep forelimb slips on the skilled ladder rung walking task after stroke compared to rats which received vehicle only (Fig. 2;  $p = 0.046$ ).

The current study was conducted to determine whether subcutaneous treatment with the isoflavone daidzein would improve behavioral and anatomical outcomes after stroke. Rats treated subcutaneously with daidzein beginning at the time of stroke exhibited significantly reduced behavioral deficits over the 14 day post-stroke study period compared with rats which received only vehicle. This is consistent with our previous findings in rats fed diets containing soy protein isolate as the sole protein source for two weeks prior to stroke [4]. This outcome was anticipated, since soy protein isolate contains daidzein, as well as other bioactive compounds. Additional studies are needed to determine the post-stroke



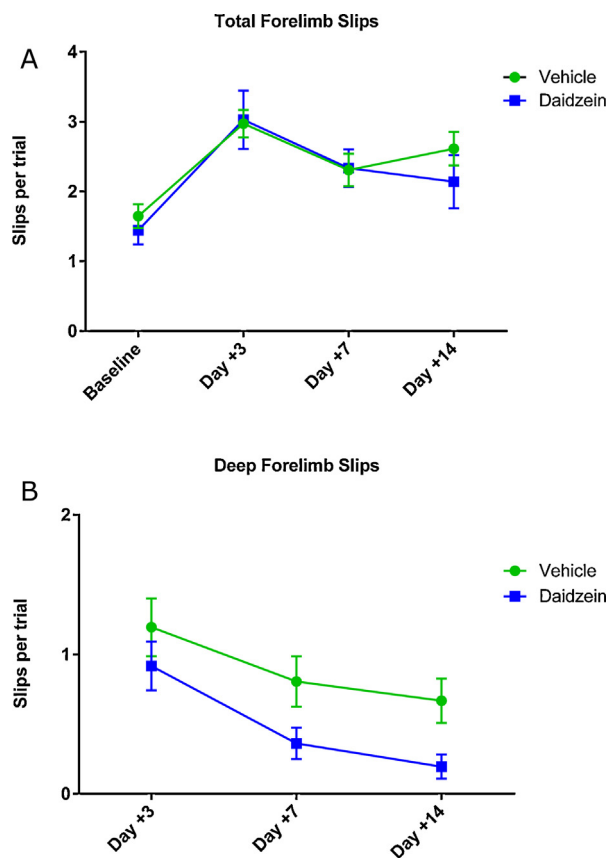
**Fig. 1.** Lesion volume and animal mass. (A) Lesion volume was quantified at the end of the study from cresyl violet-stained sections and is expressed as the percent reduction in the volume in the injured hemisphere compared to the uninjured, intact hemisphere. No difference was detected between the lesion volumes in the daidzein-treated group compared to the group which received vehicle alone ( $p > 0.05$ ). (B) Mass was recorded on Day 0, Day +3, Day +7, and Day +14. No differences were detected between groups ( $p > 0.05$ ).

window during which daidzein administration is effective, since delayed administration is likely to be more clinically relevant.

No lesion volume differences were detected in the current study, which is also consistent with our previous dietary study [4]. Further, stroke lesions in the current study were similar with previously published cases in their location (i.e. areas affected), shape, and volume [4]. We did not quantify cell survival in peri-infarct cortex in the current study. Future studies are needed to determine whether a difference exists in post-stroke neuronal survival between groups.

Although the precise mechanism(s) for the recovery of function seen in the current study remain unclear, there are several possible hypotheses. First, it is possible that the effects are the result of daidzein binding to the estrogen receptors ER- $\beta$  and/or ER- $\alpha$  in the brain, as suggested previously by others [7,9,10]. However, the estrogenic activity of daidzein is likely insufficient to solely explain its ability to reduce inflammation and protect cells in the post-stroke cerebral cortex from injury [12], even though estradiol is known to be neuroprotective [16]. Daidzein and other isoflavones bind to estrogen receptors with much lower affinity than estradiol, which likely limits the activation of pathways involved in estradiol-mediated neuroprotection [7]. Indeed, activation of estrogen receptors by estradiol does not appear to be sufficient to produce all of the downstream outcomes of isoflavone administration *in vitro* [12].

Second, it is possible that arginase (ARG1) – an enzyme which converts L-arginine to urea and ornithine and is linked to axonal plasticity and neuroprotection [17] – plays a role in mediating the observed recovery of function in rats treated with daidzein. Daidzein has been shown to induce expression of ARG1, which was associated with an increase in axonal plasticity in several *in vivo*



**Fig. 2.** Skilled ladder rung walk. (A) No difference was detected in the total number of forelimb slips over the course of the study, indicating that gross motor movements were not differently affected between groups ( $p > 0.05$ ). (B) However, deep forelimb slips – an indicator of skilled forelimb use rather than gross motor function – were found to be significantly different between groups after stroke, with the daidzein-treated rats demonstrating significantly lower deficits ( $p = 0.046$ ).

and *in vitro* models [12]. Whether ARG1 expression is induced by daidzein treatment after stroke in our model remains to be determined.

Thirdly, some of the observed neuroprotection may result from daidzein binding to peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ). The anti-diabetic thiazolidinedione drug Rosiglitazone (a PPAR $\gamma$  specific ligand) has been demonstrated to provide neuroprotection after stroke in rats [18–20]. Increases in PPAR $\gamma$  expression result in upregulated expression of 14-3-3 $\epsilon$ , superoxide dismutase (SOD1), and catalase, which work through converging pathways to reduce inflammation and apoptotic cell death [18]. Research conducted in models of other organ systems has demonstrated the ability of daidzein to bind to and activate PPAR $\gamma$  in rats [21,22].

These three hypothetical mechanisms are not mutually exclusive, and some or all of these pathways could play a role in mediating the observed positive effects of daidzein administration after stroke. Indeed, daidzein may act simultaneously through parallel and convergent pathways to exert neuroprotective effects, and activation of each of these pathways may contribute to functional improvement in a given subject. These questions must be addressed in future studies. Studies of stroke in mice genetically lacking one or more of the above factors may be necessary to address these questions.

Knowledge is also lacking regarding the identity of the cell types on which daidzein acts to mediate neuroprotection and functional recovery following stroke. Although *in vitro* experiments have determined that daidzein acts on cultured neurons to reduce

injury and increase sprouting [12], all of the other cells in the brain are likely exposed to daidzein as well, and their roles in mediating protection from stroke must also be considered. For example, ER- $\beta$  [23] and PPAR $\gamma$  [24] are expressed in the brain by glia as well as neurons. This widespread distribution increases the likelihood of non-neuronal effects which may influence stroke outcomes, including immunomodulation due to microglial activation [24]. Further, daidzein has been demonstrated to induce possible cardiovascular and cerebrovascular benefits [14], which could possibly increase perfusion and play a role in mediating improved post-stroke outcomes. Also, other systemic effects of daidzein may contribute to the observed effects, including changes in liver function and fatty acid metabolism due to activation of promiscuous nuclear receptors [25], which should also be considered in future studies using stroke models.

Herein, we have demonstrated that subcutaneous administration of the isoflavone daidzein for two weeks beginning at the time of stroke resulted in a significant increase in functional recovery on the skilled ladder rung walking task. Therefore, subcutaneous delivery of daidzein represents an effective route for administration of this compound following stroke in rats.

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