Correction of behavioral deficits and reduction of inflammation under the influence of genistein in R6/1 mice: **comprehensive research on a new therapeutic approach in Huntington disease**

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Introduction

Huntington disease (HD) is one of genetic neurodegenerative disorders. It is caused by a mutation in the *IT15* gene which consists of expansion of the CAG triplet. This expansion causes appearance of a long stretch of glutamine residues (polyQ region) in the amino acid sequence of the huntingtin protein (HTT). Mutant huntingtin (mHTT) forms aggregates which accumulate in cells, causing their dysfunctions. Unfortunately, treatment of HD consists only on alleviating its symptoms to date. One of the most promising strategy for its treatment is stimulation of mutant huntingtin degradation, particularly by the autophagy process.

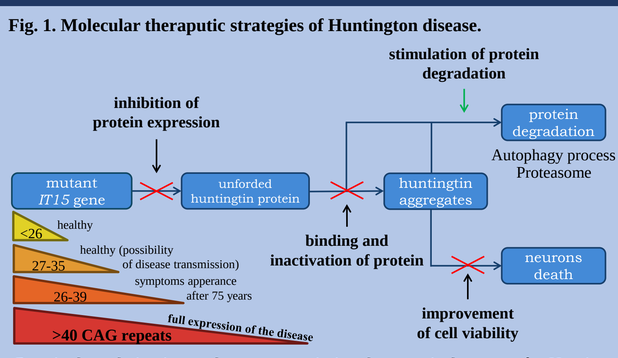
Genistein is one of the natural substances belonging to the isoflavone group and found in large quantities in legumes. Its molecular mechanism of action is based on inhibition of mTOR kinase activity resulting in the activation of lysosomal biogenesis factor (TFEB; Transcriptional Factor EB) and as a consequence, induction of the autophagy process. Given that this compound is completely safe to use (completed safety phase of clinical trials) and that it crosses the blood-brain barrier, genistein is one of the candidates for a drug that can suppress the effects and cause of HD.

Aim of the studies

The aim of the presented research was to investigate the effect of genistein on:

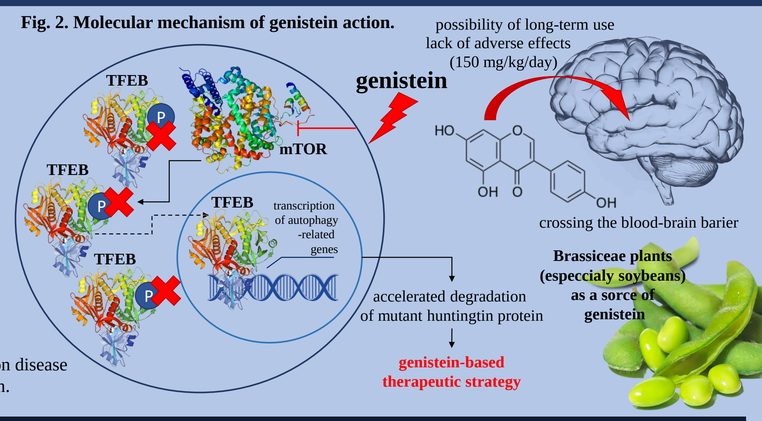
- ✓ the level of aggregates of mutant huntingtin (mHTT) on a model of cells taken from HD patients;
- ✓ cognitive and anxiety behavior as well as muscle grip strength in R6/1 mice constituting a genetically modified HD model;
- ✓ intensity of peripheral inflammation characteristic of the studied disease in R6/1 mice.

Fig. 1. Molecular theraputic strategies of Huntington disease.



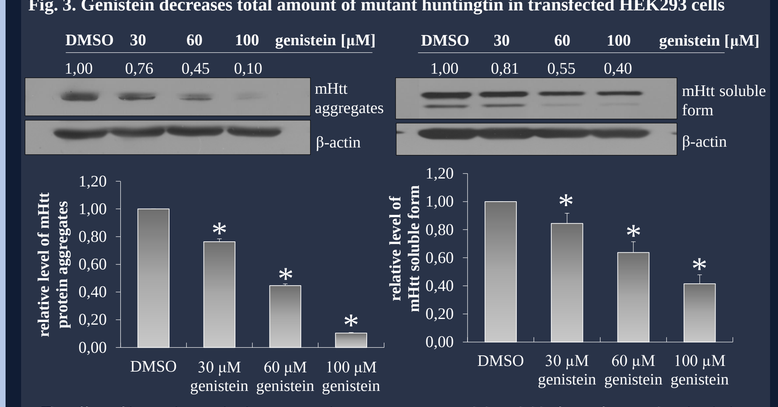
Protein degradation is one the most promissing therapeutical strategy for Huntington disease and the autophagy process is one of processes which can be use to this phenomenon.

Fig. 2. Molecular mechanism of genistein action.



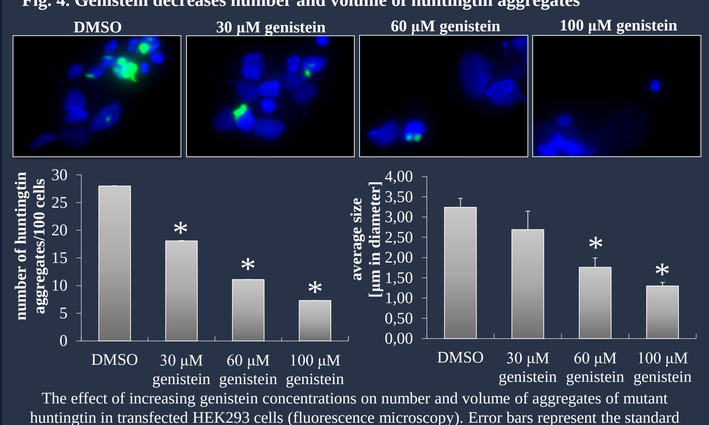
Cellular studies' results

Fig. 3. Genistein decreases total amount of mutant huntingtin in transfected HEK293 cells



The effect of increasing genistein concentrations on aggregates and the soluble form of mutant huntingtin in transfected HEK293 cells (Western blotting). Error bars represent the standard deviation. Statistical analysis was performed using t-Student's test. Differences were considered statistically significant (*) at p <0.05.

Fig. 4. Genistein decreases number and volume of huntingtin aggregates



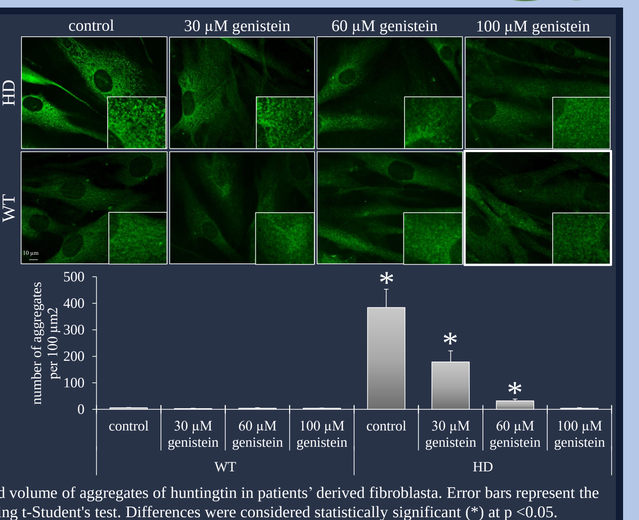
The effect of increasing genistein concentrations on number and volume of aggregates of mutant huntingtin in transfected HEK293 cells (fluorescence microscopy). Error bars represent the standard deviation. Statistical analysis was performed using t-Student's test. Differences were considered statistically significant (*) at p <0.05.

Fig. 5. Genistein decreases the level of huntingtin in patients' derived fibroblasts

Patiens' cell lines characteristic

patient type	sex	age (years)	CAG repeats number	rating scale CGI*	rating scale TFC*
C1	M	51	NT	NA	-
C2	M	50	NT	NA	-
C3	M	51	NT	NA	-
C4	M	43	NT	NA	-
HD1	M	41	43	3	II
HD2	W	45	43	4	II
HD3	M	54	43	5	III
HD4	M	49	42	4	I

*CGI - the Clinical Global Impressions scale, TFC - the Total Functional Capacity scale, NT - not tested, NA – not applicable, M – male, F - female



The effect of increasing genistein concentrations on number and volume of aggregates of huntingtin in patients' derived fibroblasta. Error bars represent the standard deviation. Statistical analysis was performed using t-Student's test. Differences were considered statistically significant (*) at p <0.05.

Rodent model results

Model of the studies:
genetically modified mouse model B6-R6/1:

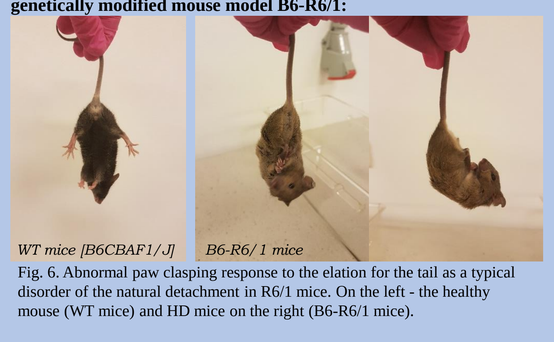


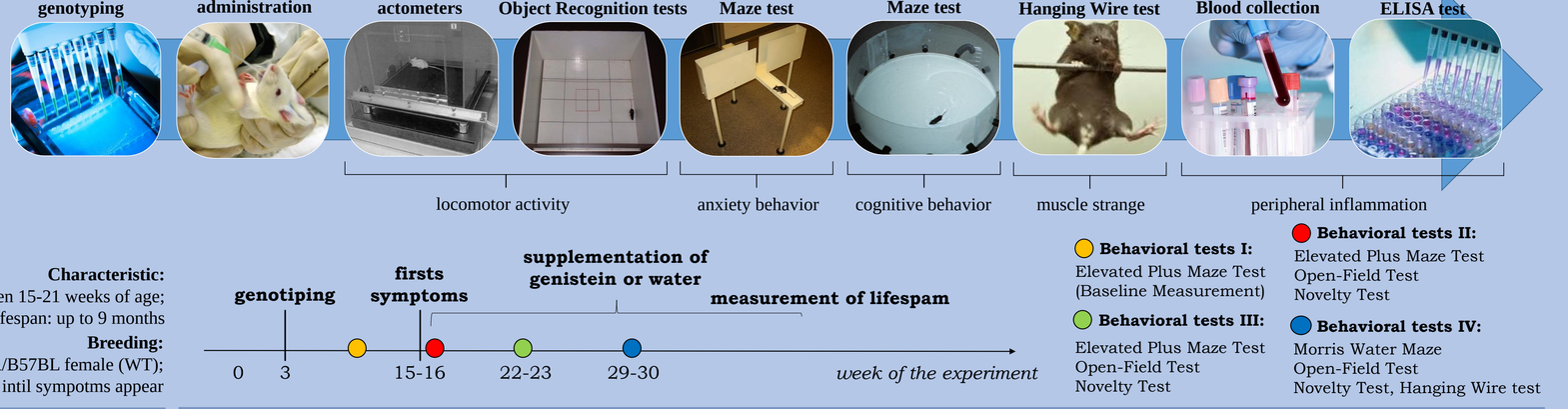
Fig. 6. Abnormal paw clasping response to the elation for the tail as a typical disorder of the natural detachment in R6/1 mice. On the left - the healthy mouse (WT mice) and HD mice on the right (B6-R6/1 mice).

symptoms

- mHTT aggregates in cells
- atrophy in striatum mass
- lost of neurons from the striatum region
- reducing the weight of the brain
- microglia activation (neuroinnflamation)
- eating disorders (weight loss)
- impaired motor coordination
- convulsions
- loss of balance
- cognitive disorders
- hyperactivity
- shortening the life span

Genetic background:
Human exon 1 of the *IT15* gene containing 115-150 CAG repeats conditioning the rapid progression of the HD.

Characteristic:
First symptoms: between 15-21 weeks of age;
Lifespan: up to 9 months
Breeding:
R6/1 male x BCA/B57BL female (WT);
possible intil sympotms appear



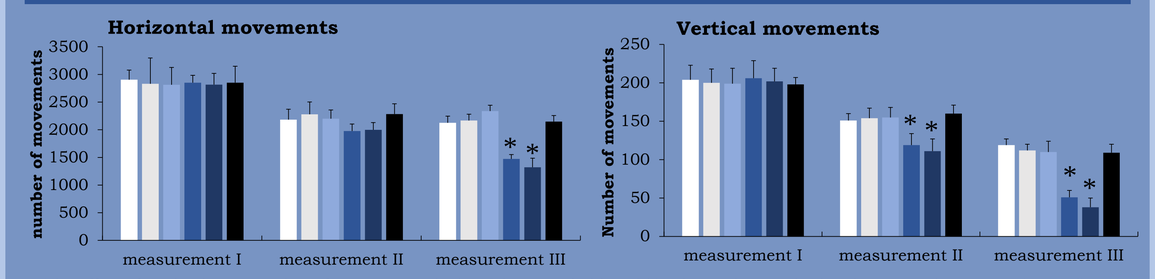
Behavioral tests I:
Elevated Plus Maze Test
(Baseline Measurement)

Behavioral tests II:
Elevated Plus Maze Test
Open-Field Test
Novelty Test

Behavioral tests III:
Elevated Plus Maze Test
Open-Field Test
Novelty Test

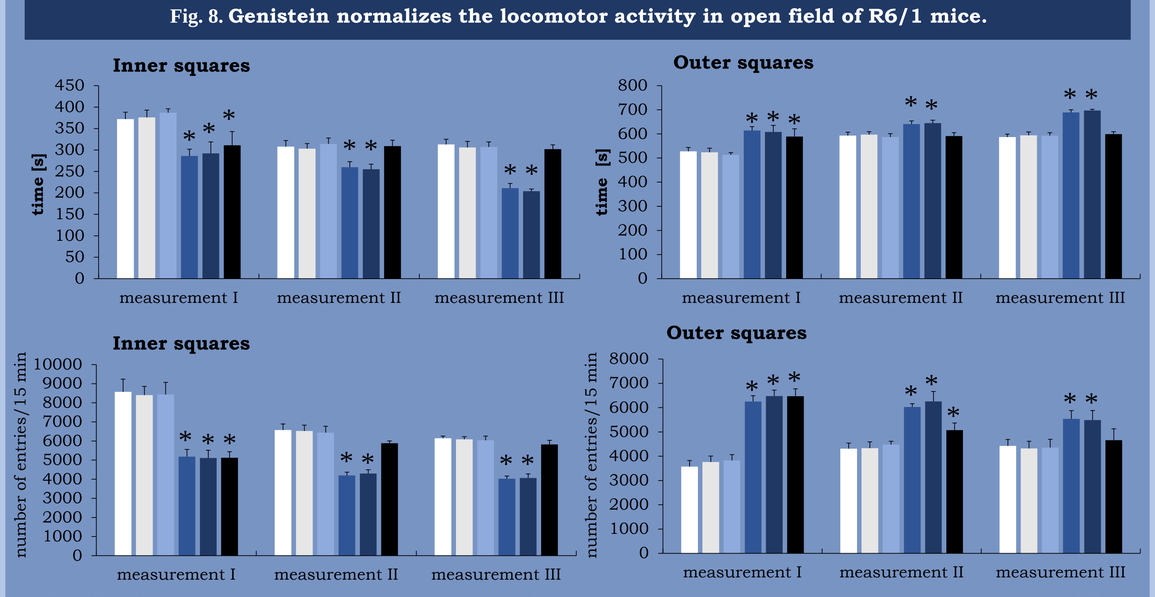
Behavioral tests IV:
Morris Water Maze
Open-Field Test
Novelty Test, Hanging Wire test

Fig. 7. Genistein normalizes the number of movements in R6/1 mice.



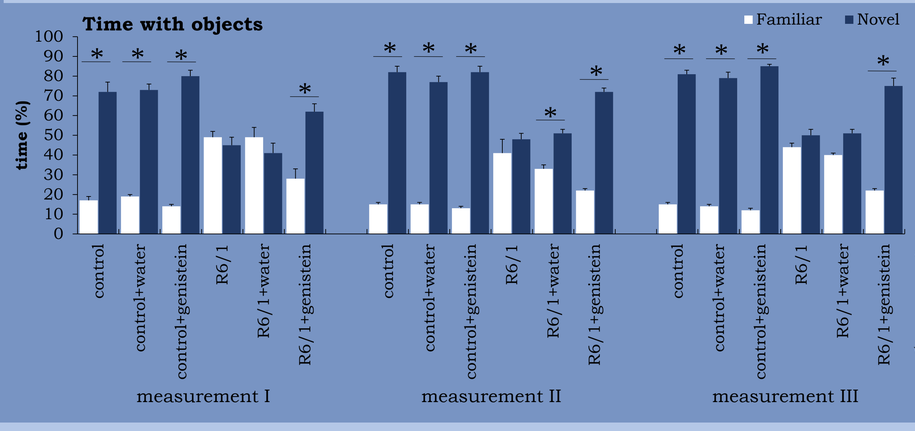
The effect of genistein on number of movements during 2 hour period in R6/1 mice and control mice that were given water (control) or genistein at a dose of 150 mg/kg/day. The tests were performed using actometers cages and the number of horintal and vertical movements were measured. Error bars represent the standard deviation. Statistical analysis was performed using ANOVA test. Differences were considered statistically significant (*) at p <0.05.

Fig. 8. Genistein normalizes the locomotor activity in open field of R6/1 mice.



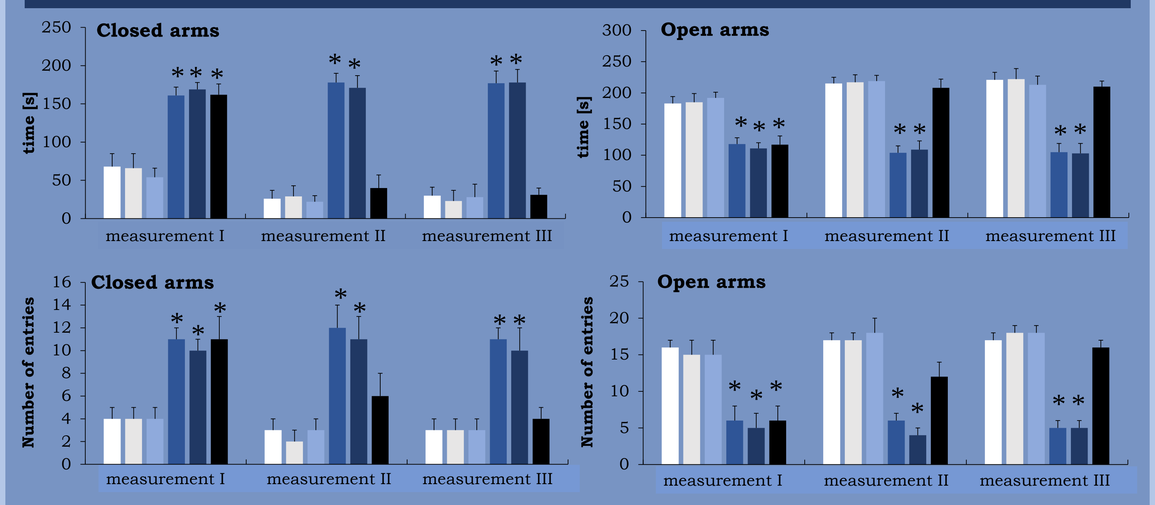
The effect of genistein on locomotor activity in R6/1 mice and control mice that were given water (control) or genistein at a dose of 150 mg/kg/day. The tests were performed using Open Field Test and the time spend in inner and outer squares as well as number of entries to inner or outer squares were measured. Error bars represent the standard deviation. Statistical analysis was performed using ANOVA test. Differences were considered statistically significant (*) at p <0.05.

Fig. 8. Genistein restores innate curiosity of R6/1 mice.



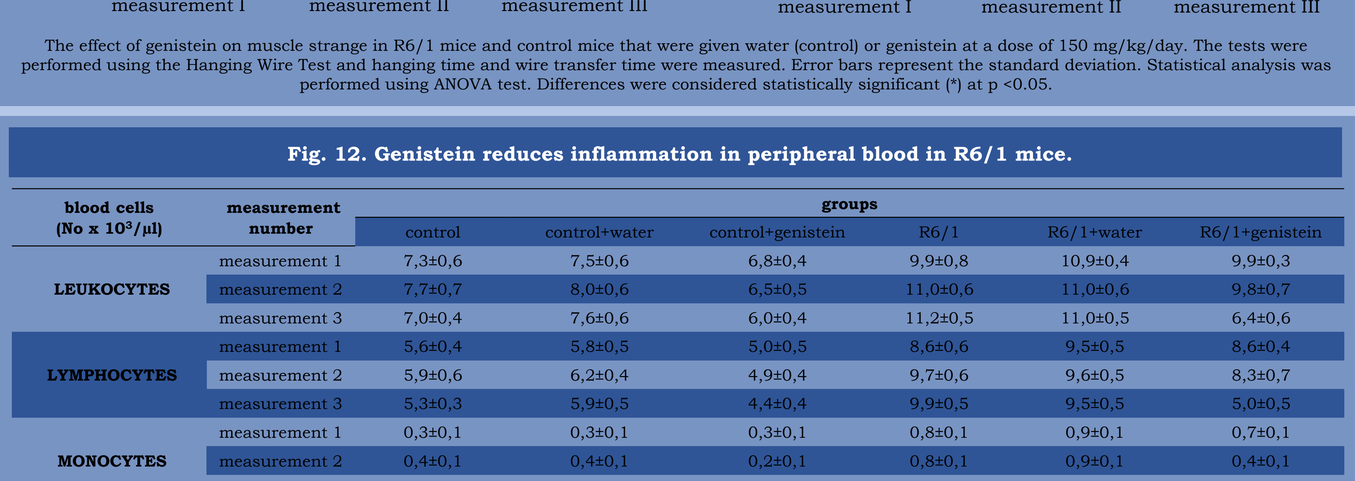
◄ The effect of genistein on innate curiosity in R6/1 mice and control mice that were given water (control) or genistein at a dose of 150 mg/kg/day. The tests were performed using Novel Objects Recognition test immediately after Open Field test and the time spend in area of familiar and novel objects were measured. Error bars represent the standard deviation. Statistical analysis was performed using ANOVA test. Differences were considered statistically significant (*) at p <0.05.

Fig. 9. Genistein eliminates anxiety disorders in R6/1 mice.



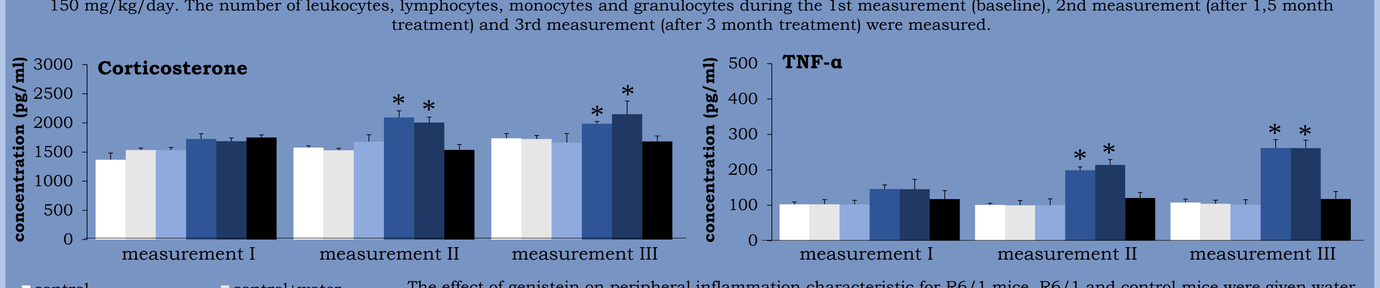
The effect of genistein on anxiety behavior in R6/1 mice and control mice that were given water (control) or genistein at a dose of 150 mg/kg/day. The tests were performed using the elevated plus maze test and the following parameters were tested: time spent in open arms (A), time spent in closed arms, number of entries to closed arms and number of entries to open arms.

Fig. 10. Genistein improves memory parameters in R6/1 mice.



The effect of genistein on memory in R6/1 mice and control mice that were given water (control) or genistein at a dose of 150 mg/kg/day. The tests were performed using the Morris Water Maze and the latency to platform and time spend in critical quadrant as well as key parameters duing the probe test are presenting in the figure. Error bars represent the standard deviation. Statistical analysis was performed using ANOVA test. Differences were considered statistically significant (*) at p <0.05.

Fig. 11. Genistein improves muscle strange in R6/1 mice.



The effect of genistein on muscle strange in R6/1 mice and control mice that were given water (control) or genistein at a dose of 150 mg/kg/day. The tests were performed using the Hanging Wire Test and hanging time and wire transfer time were measured. Error bars represent the standard deviation. Statistical analysis was performed using ANOVA test. Differences were considered statistically significant (*) at p <0.05.

Fig. 12. Genistein reduces inflammation in peripheral blood in R6/1 mice.

blood cells (No x 10^3/μl)	measurement number	control	control+water	control+genistein	R6/1	R6/1+water	R6/1+genistein
LEUKOCYTES	measurement 1	7,3±0,6	7,5±0,6	6,8±0,4	9,9±0,8	10,9±0,4	9,9±0,3
	measurement 2	7,7±0,7	8,0±0,6	6,5±0,5	11,0±0,6	11,0±0,6	9,8±0,7
	measurement 3	7,0±0,4	7,6±0,6	6,0±0,4	11,2±0,5	11,0±0,5	6,4±0,6
LYMPHOCYTES	measurement 1	5,6±0,4	5,8±0,5	5,0±0,5	8,6±0,6	9,5±0,5	8,6±0,4
	measurement 2	5,9±0,6	6,2±0,4	4,9±0,4	9,7±0,6	9,6±0,5	8,3±0,7
	measurement 3	5,3±0,3	5,9±0,5	4,4±0,4	9,9±0,5	9,5±0,5	5,0±0,5
MONOCYTES	measurement 1	0,3±0,1	0,3±0,1	0,3±0,1	0,8±0,1	0,9±0,1	0,7±0,1
	measurement 2	0,4±0,1	0,4±0,1	0,2±0,1	0,8±0,1	0,9±0,1	0,4±0,1
	measurement 3	0,3±0,1	0,4±0,1	0,2±0,1	0,8±0,1	0,8±0,1	0,4±0,1
GRANULOCYTES	measurement 1	0,3±0,004	0,3±0,004	0,2±0,003	0,5±0,006	0,5±0,003	0,4±0,005
	measurement 2	0,3±0,002	0,3±0,004	0,3±0,003	0,6±0,004	0,6±0,004	0,4±0,004
	measurement 3	0,3±0,003	0,3±0,004	0,3±0,002	0,6±0,004	0,6±0,003	0,3±0,003

The effect of genistein onselected morphological elements of peripheral blood in R6/1 mice and control mice that were given water (control) or genistein at a dose of 150 mg/kg/day. The number of leukocytes, lymphocytes, monocytes and granulocytes during the 1st measurement (baseline), 2nd measurement (after 1,5 month treatment) and 3rd measurement (after 3 month treatment) were measured.

The effect of genistein on peripheral inflammation characteristic for R6/1 mice. R6/1 and control mice were given water (control) or genistein at a dose of 150 mg/kg/day. The tests were performed using ELISA and concentretion of corticosterone and TNF-α were measured. Error bars represent the standard deviation. Statistical analysis was performed using ANOVA test. Differences were considered statistically significant (*) at p <0.05.

Conclusion

Genistein reduces the level of soluble as well aggregates of mutant huntingtin protein.
Genistein corrects abnormal locomotory activity, anxiety behawior, cognitive behawior and reduced muscle strange in mice model of Huntington disease.
Genistein reduces peripheral inflammation characteristic for Huntington disease.

Considering that genistein is a safe compound that crosses the blood brain barier, it is a viable candidate for a Huntington disease' drug.

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