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# Effect of *Coturnix japonica* (quail) egg yolk in ethanol damage-induced mice

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## ABSTRACT

**Introduction:** The high prevalence of neurodegenerative diseases in the older adult population requires research focused on functional foods with regulatory properties on redox state and with antioxidant potential. Quail egg yolk is a food with a great diversity of antioxidant compounds with neuroprotective activity.

**Objective**: To evaluate the effect of *Coturnix japonica* egg yolk administration on the nervous tissue of mice against ethanol damage induction.

**Methodology:** 35 mice received the following treatment via orogastric for five days: groups I and II water (10 mL/kg), group III egg yolk (5 mL/kg), group IV egg yolk (10 mL/kg) and group V egg yolk (15 mL/kg). On the fifth day, 99% ethanol was administered subcutaneously (5 g/kg) to groups II-V. After four hours, the mice were decapitated to obtain the brain and cerebellum and subsequently perform biochemical tests and histopathological analysis.

**Results:** Group IV presented neuronal proliferation phenomenon and Purkinje cells pluristratification in the brain and cerebellum respectively and additionally presented a better GSH/GSSG ratio ($p<0.05$) in comparison to group II. There was no statistically significant difference between protein levels or protein sulfhydryl groups in any of the groups.

**Conclusions:** *Coturnix japonica* egg yolk administration resulted in better preservation of the brain and cerebellum's cytoarchitecture and increased GSH profile.

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## KEYWORDS

Nervous tissue, egg yolk, glutathione, protein, mice, quail.

## ABBREVIATIONS

ND: Neurodegenerative diseases.

AD: Alzheimer's disease.

PD: Parkinson's disease.

PUFA: Polyunsaturated fatty acids.

iNOS: Inducible nitric oxide synthase.

PPAR: Perosyxomel proliferator-activated receptors.

HE: Hematoxylin-eosin.

GSH: Reduced glutathione.

SH: sulfhydryl groups.

PCs: Purkinje cells.

DHA: Docosahexanoic acid.

EAAC1: Excitatory amino acid transporter.

EY: Egg Yolk.

NOX: NADPH Oxidases.

GSSG: Oxidized glutathione.

## INTRODUCTION

Neurodegenerative diseases (ND) are defined as incurable and debilitating conditions that result in the progressive degeneration and death of nerve tissue cells, leading to problems such as dementia[1]. Among the NDs, Alzheimer's is the most prevalent dementia spectrum worldwide[2], according to Global Burden Disease an estimated 152 million people will suffer from Alzheimer's and other dementias[3].

Worldwide, Alzheimer's disease (AD) and Parkinson's disease (PD) mostly affect the elderly, which may become more important given the progressive increase in the population over 65 years of age[4]. Currently, according to data from the National Institute of Statistics and Informatics (INEI), it is estimated that by 2050 the population over 65 years of age will represent 18.3% of the total population[5].

In this situation, it has been observed that the consumption of functional foods is associated with a reduced risk of neurodegenerative diseases[6], which could be explained by the presence of nutrients such as selenium, polyunsaturated fatty acids (PUFA), vitamin E and lutein, which may exert regulatory roles on enzymes and inflammatory pathways such as inducible nitric oxide synthase (iNOS), caspase-9, Bcl-2, CD36 expression, PPAR activation, among others[7]. Egg yolk (EY) is a food matrix that has been shown to contain predominantly vitamin E, PUFA, and selenium[8], therefore, there is biological plausibility to support a neuroprotective effect.

The results obtained could broaden the body of knowledge about the functional effects that egg yolk can exert, considering that effects in other physiological areas have already been reported[9,10]. Given that this food contains a diverse variety of nutrients with neuroprotective roles, this study can serve as a basis for establishing future nutritional synergy studies among them, which could lead to the generation of nutraceutical products with the appropriate proportions to observe functional effects.

The present study aims to evaluate the effect of *Coturnix japonica* egg yolk administration on the nervous tissue of mice against ethanol damage induction.

## MATERIALS AND METHODS

**Research design:** The present study is purely experimental, with a post-test and a control group[11].

**Quail egg yolk obtention:** The product was obtained from the AVICOD Poultry Production Center in Peru, the egg yolk was manually separated and placed in a beaker for subsequent administration with an orogastric cannula, this procedure was performed for each day of treatment.

**Evaluation of the neuroprotective effect:** 35 adult male albino Mus musculus *Mus musculus* strain BALB/c mice obtained from the National Institute of Health biotherium, weighing 30.5 ± 1.4 g, were used. The animals received a balanced diet obtained from the National Agrarian University of La Molina, and the experimental units were acclimatized for seven days with 12-hour light-dark cycles.

After the acclimatization period, the mice were randomly distributed into five groups (n=7), which received the following treatment via orogastric for five days:

- Group I - Negative Control: Water 10 mL/kg
- Group II - Positive control: Water 10 mL/kg
- Group III - Experimental 1: Egg yolk 5 mL/kg
- Group IV - Experimental 2: Egg yolk 10 mL/kg
- Group V - Experimental 3: Egg yolk 15 mL/kg

On the fifth day, after completing treatment administration, ethanol (99%) at a dose of 5 g/kg was immediately applied to groups II-V, a technique used by Ali[12], while group I received physiological saline (NaCl 0.9%), both subcutaneously. After exactly four hours had elapsed, the animals were sacrificed by immediate decapitation, subsequently, a craniotomy was performed to extract the brain and cerebellum, which were washed with NaCl 0.9%, to be subsequently weighed on an analytical balance (Sartorius ®), The left hemisphere and cerebellum were placed in 10% formalin in phosphate buffer 0.075 mol/L pH 7.4 for subsequent histological analysis, while the right hemisphere was used for the preparation of the homogenate and subsequent biochemical analysis.

### *Histopathological evaluation*

The left hemispheres of the cerebrum and cerebellum were preserved in 10% formaldehyde in phosphate buffer at 0.075 mol/L pH 7.4; they were then fixed in kerosene and stained with hematoxylin-eosin (HE) by a Medical Technology professional. The readings of the slides were performed by a Pathologist, both professionals from the Institute of Legal Medicine and Forensic Sciences. The analysis considered the preservation of the layers of the brain and cerebellum, level of cellular abundance, presence of eosinophilia, and presence of inflammatory components[13].

### *Determination of biochemical indicators*

- **Nerve tissue homogenization:** The right hemispheres had an average weight of 0.164 g, this was diluted with phosphate buffer 0.01 mol/L pH 7.4 at a ratio of 1:10, then centrifuged at 3000 revolutions per minute (RPM) for 5 min to obtain the supernatant of the homogenate for biochemical tests.
- **Determination of GSH profile:** The method of Ellman (1959) with modifications of Boyne (1972) was followed, where glutathione levels were determined by reaction with DTNB. A deproteinized product was prepared by placing 950 µL of supernatant with 50 µL of 100% TCA and centrifuged at 13 000 rpm for 5 minutes. From the resulting supernatant, 300 µL was combined with 1.5 mL of phosphate buffer pH 6.8 0.5 mol/L. For the determination of total GSH, the reducing effect of ascorbic acid and glyoxylic acid at a 2:1 ratio on the oxidized GSH fraction (GSSG) was used, samples were placed in a water bath at 67°C for 5 minutes, then were left to cool and 0.3 mL of DTNB diluted in 0.5 mol/L phosphate buffer at a concentration of 1.5 mg/mL was added. Finally, for the GSH/GSSG ratio, a division was performed to obtain a di-

mensionless ratio between GSH and GSSG values, the samples were read at 412 nm[14,15].

- **Determination of proteins and protein sulfhydryl groups:** The method described by Lowry (1951) was used, where the Folin-Ciocalteu reagent was used to measure protein levels in brain tissue, samples were read at 580 nm[16]. To measure protein sulfhydryl groups (SH), the method of Lindsay and Sedlak (1968) with modifications by Suárez (2014) was followed, where the levels of protein thiol groups were determined. For this purpose, 0.1 mL of supernatant from the homogenate was diluted and combined with 900 µL of buffer pH 7.4 0.01 mol/L and placed in a water bath at 37°C for 5 minutes, then DNTB was added following the steps explained in the GSH protocol, the samples were read at 412 nm.[14,17].

### *Statistical analysis*

The Shapiro-Wilk normality test was performed (n<50); the protein and protein sulfhydryl groups indicators presented normal distribution, for which the analysis of variance test (ANOVA) was applied, then the Levene homoscedasticity test, given that the indicators presented non-homogeneous variances, for which the Games-Howel test was applied. The total GSH and GSH/GSSG indicators presented an asymmetric distribution, to which the Kruskal-Wallis test was applied, and the Mann-Whitney test with Holm–Bonferroni correction for multiple comparisons was used to make comparisons between groups. For this analysis, the IBM® SPSS Statistics 25 statistical program and the R program version 4.1.1 through the R Studio® platform were used.

### *Ethical aspects*

The protocol was approved by the Ethics and Research Committee of the Nutrition Department in the National University of San Marcos. The ethical standards and procedures were considered in accordance with Peruvian Law No. 30407, Law for the Protection and Welfare of Animals[18].

## RESULTS

It was observed that group II (received ethanol) had lower protein levels in comparison to group I. On the other hand, higher levels were observed in groups III-V compared to group II, without being significant (Table 1).

Concerning the GSH profile, it was observed that group II presented lower GSH/GSSG levels with respect to group I (p<0.01). In group IV a higher level of GSH/GSSG ratio was observed compared with group II (p<0.05); in group V it was observed that the level of these indicators did not show a significant difference with group II (Table 2). It was evidenced that group II showed a higher level of protein thiol groups with respect to group I, but it was not significant; finally, the yolk treatment groups (III-V) had a higher level of protein sulfhydryl groups (Table 1).

Among the histological findings (Figure 1) at the brain level, it was found that the mice that received ethanol (group II) evidenced cellular edema, eosinophilia, nuclear pyknosis, as well as cytoplasmic disruption, and increase of glia; as for the cerebellum, eosinophilia, and loss of nucleus in Purkinje cells (PCs) were found.

On the contrary group I presented a preserved cytoarchitecture in the brain and cerebellum. Group III presented phenomena of increase of neuronal cells in the brain, as well as alteration of the blood-brain barrier, in the cerebellum there was evidence of slight cytoplasmic retraction and slight vascular congestion. Group IV presented hypercellularity and grouping of neurons in a back-to-back conformation at the cerebral level; at the cerebellum level, pluristratification was observed in the PCs, forming double layers. On the other hand, group V presented alteration of the cytoplasmic membrane and proliferation of neurons in the brain; in the cerebellum, eosinophilia was observed in some PCs.

**Table 1.** Protein levels and protein sulfhydryl groups in mouse brain tissue according to treatment group

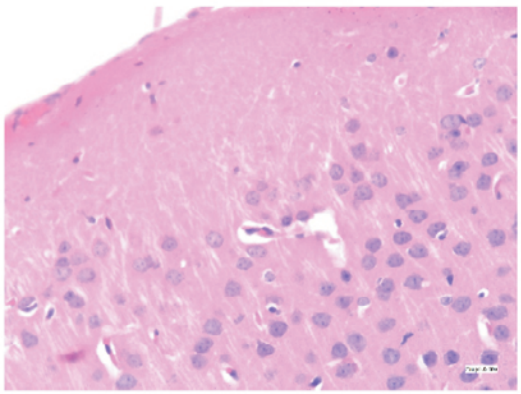
| Treatment groups | Protein* (mg/g tissue) | Protein SH* (µmol/mg protein) |
|---|---|---|
| | Mean ± SD | Mean ± SD |
| Group I: Water 10 mL/kg + NaCl | 66,36 ± 4,30 | 3,13 ± 0,87 |
| Group II: Water 10 mL/kg + ethanol | 55,74 ± 5,68 | 2,98 ± 0,32 |
| Group III: Yolk 5 mL/kg + ethanol | 66,64 ± 6,59 | 2,72 ± 0,33 |
| Group IV: Yolk 10 mL/kg + ethanol | 56,42 ± 3,58 | 3,16 ± 0,26 |
| Group V: Yolk 15 mL/kg + ethanol | 66,30 ± 8,22 | 3,18 ± 0,51 |

*Shapiro Wilk – ANOVA (p>0,05).

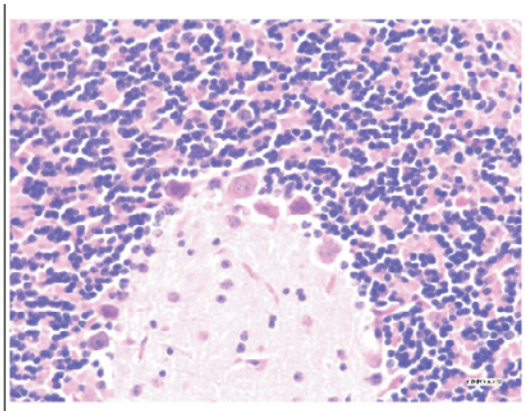
**Table 2.** GSH profile levels in mouse nervous tissue according to treatment group

| Treatment groups | GSH* (nmol/mg protein) | GSH total** (nmol/mg protein) | GSH/GSSG*** |
|---|---|---|---|
| | Mean ± SD | Median (IQR) | Median (IQR) |
| Group I: Water 10 mL/kg + NaCl | 100,3 ± 36,8 | 278,0 (60,9) | 0,64 (0,38) [a] |
| Group II: Water 10 mL/kg + ethanol | 70,0 ± 15,6 | 303,6 (42,6) | 0,31 (0,13) |
| Group III: Yolk 5 mL/kg + ethanol | 35,7 ± 6,0 [a] | 238,6 (59,7) | 0,17 (0,03) [a] |
| Group IV: Yolk 10 mL/kg + ethanol | 109,0 ± 10,4 [a] | 272,5 (65,7) | 0,82 (0,28) [b] |
| Group V: Yolk 15 mL/kg + ethanol | 57,3 ± 10,4 | 214,8 (81,1) | 0,32 (0,54) |

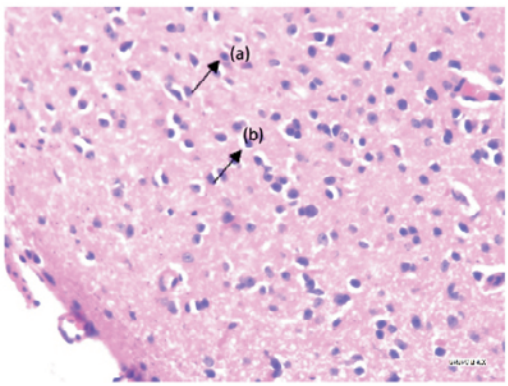
* Shapiro-Wilk - ANOVA, Levene (p<0,05), post hoc Games-Howell.
** Shapiro-Wilk – Kruskal-Wallis (p>0,05).
*** Shapiro-Wilk – Kruskal-Wallis (p<0,05), Mann-Whitney U with Holm adjustment.
(a) p<0,01 Compared to group II. (b) p<0,05 Compared to group II. SD: Standard deviation.IQR: Interquartile range.



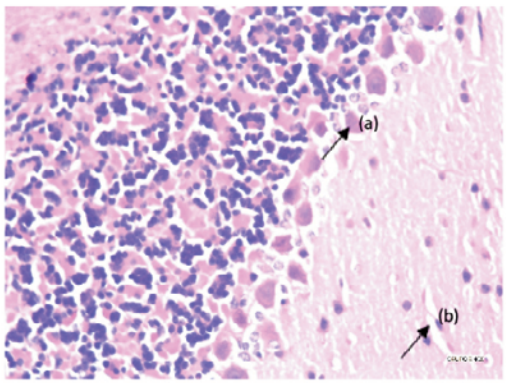
**Microphotography 1.** Group I. Brain. The morphology of the brain is appreciated without alterations. **HE**



**Microphotography 2.** Group I. Cerebellum. The morphology of the cerebellum is appreciated without alterations. **HE (40X)**

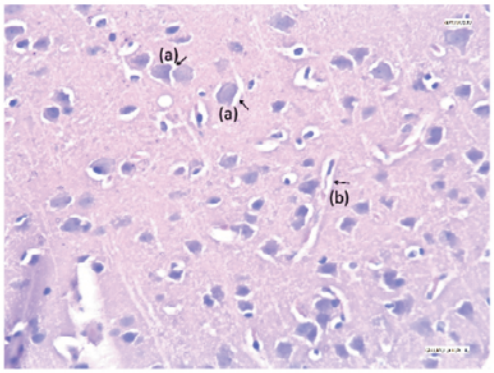



**Microphotography 3.** Group II. Brain. Increased glia, (a) nuclear pyknosis in neuronal cells (b) neuroglia. **HE (40X).**

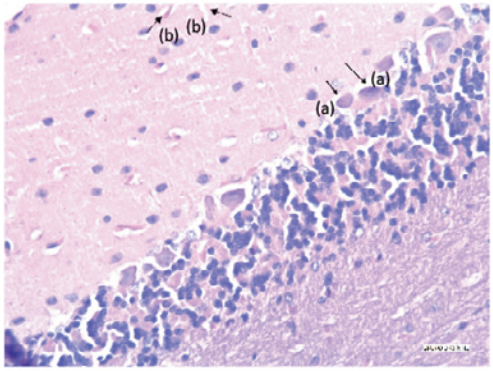



**Microphotography 4.** Group II. Cerebellum. (a) Purkinje layer with eosinophilia. (b) Molecular layer with cells in cytoplasmic retraction. **HE (40X).**

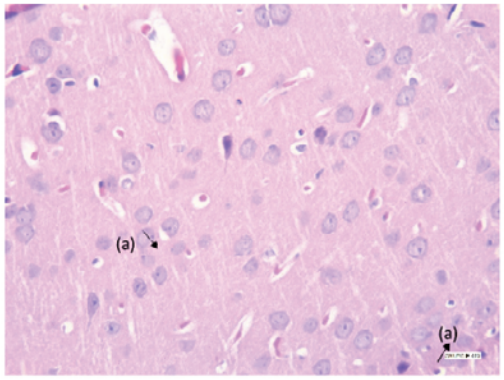
**Figure 1.** Microphotographs of control (I and II) and treatment (III-V) groups



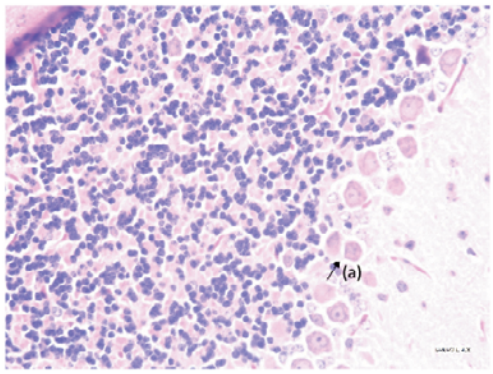

**Microphotography 7.** Group III. Brain. There is evidence of (a) Increase of neuronal cells, (b) altered blood-brain barrier. **HE (40X)**



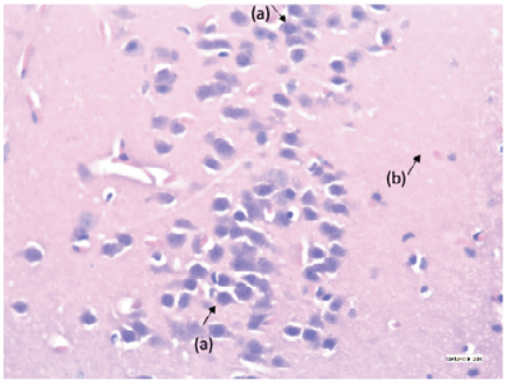

**Microphotography 8.** Group III. Cerebellum. There is evidence of (a) mild cytoplasmic retraction of Purkinje cells, (b) vessels with mild vascular congestion. **HE (40X).**



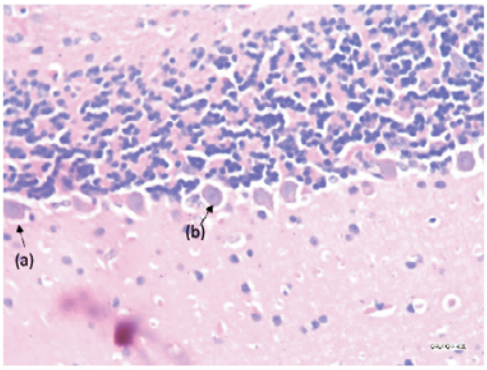

**Microphotography 9.** Group IV. Brain. (a) Hypercellularity (increase of neurons), grouped back-to-back without alterations of the cytoplasmic membrane. **HE (40X).**




**Microphotography 10.** Group IV. Cerebellum (a). Pluristratification of Purkinje cells per field (double layer). **HE (40X).**




**Microphotography 11.** Group V. Brain. (a) Focal neuronal proliferation per field (b) acellular field (absence of neuronal cells).**HE (40X).**




**Microphotography 12.** Group V. Cerebellum. (a) Purkinje cell with eosinophilia, (b) Purkinje cells preserved and grouped in a single layer.. **HE (40X).**

**Figure 1 continuation.** Microphotographs of control (I and II) and treatment (III-V) groups

## DISCUSSION

Polyunsaturated fatty acids present in EY like docosahexaenoic acid (DHA), have been shown to increase GSH levels in the face of ethanol exposure[19], this is done by increasing the expression of the excitatory amino acid transporter (EAAC1), EAAC1 stimulation would contribute to cysteine uptake and GSH synthesis[20]. This could explain the progressive increase in GSH/GSSG in groups III and IV.

On the other hand, the decreased GSH levels seen in group V could be explained by the proinflammatory role of palmitic acid and EY cholesterol. The former is known to act through the generation of ROS by inducing cytokines such as TNF-α, IL-1β, and IL-6[21]. In addition, given that NADPH would be diminished by the effect of ethanol on the NADPH Oxidases (NOX) pathway as a mechanism of ROS generation[22], then oxidized glutathione (GSSG) originated from the ascorbic acid and tocopherol cycle and could not be reduced again[23].

Protein SH group levels are maintained in a reduced state by GSH[24], this dynamic is regulated by the nuclear factor erythroid 2 pathway, the antioxidant response element (Nrf2/ARE), and glutaredoxins, the regulation of this enzyme is dependent on cellular redox state and NADPH levels[25], This could explain the tendency to observe elevated levels of protein SH groups in groups IV and V. The verification of the hypothesis about the protective role of GSH on protein SH and the inverse relationship observed in the present study with low levels of GSH and high levels of protein SH in group V goes beyond what was proposed by the present investigation.

The preservation of morphological components and absence of inflammatory components at the brain level seen mainly in group IV could be explained by the synergistic action of DHA and lutein in the activation of the MAPK/ERK pathway[26], this pathway is related to greater synaptic plasticity.

The phenomenon of back-to-back neurons and hypercellularity seen in group IV could be related to the synergy between lutein and zeaxanthin on the activation of the neural adhesion molecule (NCAM), which has the role of reorganizing the nerve cells after damage[27]. Likewise, the absence of edema in groups III-V could be explained by the inhibition of DHA on the expression of aquaporin 4 (AQ-4)[28].

Among the limitations of this study is the lack of measurement of enzymatic activity indicators such as catalase and superoxide dismutase, catalase being one of the enzymes involved in ethanol metabolism. In addition, the form of administration, which was by orogastric route, would not imply a natural form of consumption, so supraphysiological doses are likely to be reached.

## CONCLUSION

It is concluded that the administration of *Coturnix japonica* egg yolk produced a better preservation of the cytoarchitecture of the brain and cerebellum, as well as an increase in the profile of GSH, mainly at 10 mL/kg, more research is needed to elucidate more mechanisms of protection or damage at other doses.

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