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Molecular assessment of intermediate filamented protein as a detective marker of quails' histopathological brain lesions

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Abstract

The nervous system plays a crucial role in regulating most of the body's vital functions. Stress factor or infection may affect the Nervous System, which includes both the central and peripheral systems. The aims of this work are to determine the pathological lesions in quail's brain, determine the immune reactivity of GFAP proteins and RT-PCR expression of NF-KB. Fifteen samples of quail brain that showed nervous clinical signs were obtained from the affected farms. The samples were transferred to the laboratory. After the anatomical characterization, sections were cut at a thickness of 4 -5µand routine staining procedures were applied. Immunohistochemistry for GFAP protein was performed according to the company's protocol and polymerase chain reaction for NF-KB was also performed. Typically, brains histological findings showed Oligodendrocytes infiltration, perivascular cuffing, cellular adaptation, vascular changes, degeneration necrosis and architecture alteration. GFAP immunoreactivity found strong reactions inside cerebral capillary, glial cell, astrocyte, spinal cord and cerebral region. Significant increase in thickening of blood vessels percent at 23.6% and a marked decreased in gliosis percent 10.86% . The RT-PCR for the NF-KB protein showed variable degree of the expression between the all affected samples ,the highest expression was in 1and 2 samples (16.75), interestingly sample 9 and 10 showed the less expression .However the lowest expression was recorded in samples 5and 6 (18.85).The rest of samples represented by 3,4,7and 8 have close expression. We conclude that although the quail is resistant to environmental conditions, it is vulnerable to stress factors or any types of infections that effect on the efficiency of the central nervous system.

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Introduction

In vertebrates, the nervous system consists of continuously active neural progenitors that are accountable for generating new nerve cells throughout life (1). The formation of new neurons from this ancestor is named neurogenesis which includes proliferation of cells, discernment and migration and the integration of new cells into existing nerve circuits (2). In adult's mammalian brain, neurogenesis is bounded to the sub granular hippocampus zone and to the lateral ventricle sub ventricular zone (3-5). However, avian neurons are created along the lateral ventricle wall and radially migrate to be incorporated into diverse telencephalon circuits (6). Avian brain neurogenesis has been shown to be organized by several factors including level of sex steroids hormone, day length variation, age and change of season (7,8). The production and neuron cell recruitment decline with age related in finches and zebra (9). Additionally, neurogenesis was notified to descend with age in the ventricular zones of the ring doves telencephalic, olfactory bulb and the hippocampus of the pigeon (10,11) and the telencephalon of broilers (12). Mouriec and

Balthazart announce a nib decline in proliferation of cell in the medium nucleus of Japanese quails' brain through the stage of the pre-pubertal which was follow up by increasing in the proliferation of cell at puberty, the majority studies of quail's neurogenesis focused on the sensitive area of steroid sex like. Amplification of PCR and RT-PCR is considered a major technique in biology process (13-16). The activation of NF-KB transcription factor is considered as an early marker for stress of cells in different tissues such as the brain (17). NF-KB is multidirectional regulatory protein contributory in regulation genes and is related to several pathological and physiological process like proliferation of cell, inflammation, transformation, immune response (18) and apoptosis of cell (19), signaling pathway of this protein have certain impacts in the chicken immunization process against infection can significantly stimulate the secretion of NF-KB in animals' body (20,21). Two or more cytokines may have a hostile, enhanced or synergistic impacts in immunization of animals (22,23). GFAP is a Nano filament protein that is expressed by various central nervous system cell inclusive ependymal and astrocytes cell (24), this protein is meditate the help in maintains the mechanical astrocyte strength and cell shape but its main functions remnant poorly understands (25,26) in spite of the several studies which using it as a cell biomarker.

The present study aims to study lesion types, determine the proportion of Histopathological lesions, immunohistochemistry and treatise the molecular expression of NF-KB in quail's brain.

Materials and methods

Ethical approval

All animals were used to adhere to the guidelines of the Ethics Committee of the Mosul University and stratify with ethical numbers no.UM.VET.2024.083.

Animals

The study included the evaluation of histopathological lesions, immunochemistry and gene expression: The ages of the quails were 1-45 days old. Samples were collected from two fields located in Al- Rashidiyaarea area, which showed neurological signs represented by head twisting, paralysis and head asymmetry, with a moderate -high mortality rate. Samples were collected by the farm's supervising doctor then affected samples were sent to the laboratory.

Collection and processing of brain tissue

After the sacrifice of animals, the head was separated from the body. Appropriate representative samples from brains in the dimension of 2-3 cm were fixed in 10% of buffered formalin, desiccated by alcohol, purified by xylol, later the section in paraffin wax were embedded. Histological blocks of paraffin are sliced at 3-4 micrometers by microtome, stained with routine stains (Hematoxylin and Eosin). The Histopathological findings were assorted into three stage awards to their severity (27) (Table 1).

Glial fibrilly acidic protein detection

To reveal GFAP the following steps was used, paraffin removed from slices , hydrate for 20 min with tap water ,handling for 15 min by 3% H_2O_2 and wash with distal water, at room temperature retrieval of antigen was done for 5 min by proteinase K ,wash with distal water followed by phosphate buffer saline, by 5%of bovine serum albumin the slices were blocking in microwave for 5 min at 250w, with primary Ab (polyclonal rabbit Anti -GFAP, Dako, USA) and diluted for 30 min (1 in 500) the tissue incubated with primary Ab, ulterior washing, the section incubated with a secondary reagent at room temperature for 30 min after washing, the substrate was added (3,3 diaminobenzidine),counterstained with Harris Hematoxylin and covered under Dibutylphalate polystyrene Xylene (28-30).

Table 1 : Classification and stage of Histopathological lesions severity of quails brain

Stage	Categories	Description of lesion
Miner	Inflammation	Gliosis and Oligodendrocytes infiltration, perivascular cuffing
Moderate	Cellular adaptation	Hypertrophy of astrocyte
	Vascular changes	Vascular hemorrhage, edema and blood vessels congestion
Sever	Degeneration necrosis	Liquefactive necrosis, meningitis desquamation, spongy changes and axonal sphenoid

RNA extraction based on kit instructions (Addbio, Korea)

A frozen brain tissue sample (50-100 mg) was collected and placed in microcentrifuge tube. The sample was homogenized by adding 800 µl of lysis buffer, 4 µl of ß-mercapto-ethanol, and 20 µl of proteinase K solution (20 mg/ml) to a 1.5 ml microcentrifuge tube, followed by vortexing. The mixture was centrifuged at 13,000 rpm for 3 minutes. The supernatant was carefully transferred to the white ring column centrifuged at 13,000 rpm for 30 seconds. The flow-through was saved. The supernatant (500-600 µl) was transferred to a new 1.5 ml microcentrifuge tube, and 200 µl of absolute ethanol was added. The lysate (600 µl) was transferred to the green ring column with a 2.0 ml collection tube and centrifuged at 13,000 rpm for 10 seconds. The flow-through was discarded. Then, 500 µl of washing solution 1 was added to the spin column and centrifuged at 13,000 rpm for 10 seconds. Additionally, 700 µl washing

solution 2 was added to the spin column with the collection tube and centrifuged at 13,000 rpm for 1 minute. The spin column was dried by additional centrifugation at 13,000 rpm for 1 minute to remove residual ethanol. The spin column was transferred to a new 1.5 ml microcentrifuge tube, and 50 µl of elution solution was added to the spin column with the micro centrifuge tube and left to stand for at least 1 minute. The total RNA eluted by centrifugation at 13,000 rpm for 1 min. The extracted RNA is stored at -20°C.

Expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) Gene

The Korean company Addbio supplied the RT-PCR SYBR Master kit. Applications requiring reverse transcription of RNA into cDNA and subsequent real-time PCR amplification are the focus of the One RT-PCR SYBR Master Mix. The Master mix was just supplemented with primers and sample RNA. 5' AAT CAC AAA GCA GGC AGA GG -3′ and 5'- TGC CTT CTG ATG CTT GAG GA -3′ were the intended sequences for the NF-kB forward and reverse primers, respectively. Macrogen, a Korean company, synthesized each primer. One RT-PCR SYBR Master Mix, 0.5 µl of each forward and reverse primer, 3 µl of RNA template, and 6 µl of nuclease-free water made up the entire assay volume of 20 µl. Reverse transcription at 50°C for 20 min, RNA hybrid denaturation and reverse transcriptase inactivation at 95°C for 10 min, and forty cycles of qPCR with denaturation at 95°C for 15 sec. and annealing at 60°C for 60 sec. were the ideal reaction conditions, per the manufacturer's instructions. The StepOnePlus Real-Time PCR apparatus from the USA used to conduct real-time PCR (Figure 1 and 2).

Statistical analysis

Chi-Square program was used for the microscopic data analyses at P<0.01 the mean values and collect standard error of the calculated traits were deliberated (31).

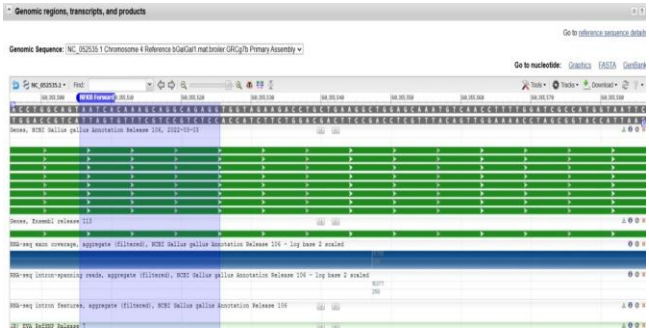

Figure 1: Nucleotide sequence of target NF-KB forward primers designed through Gen Bank database.

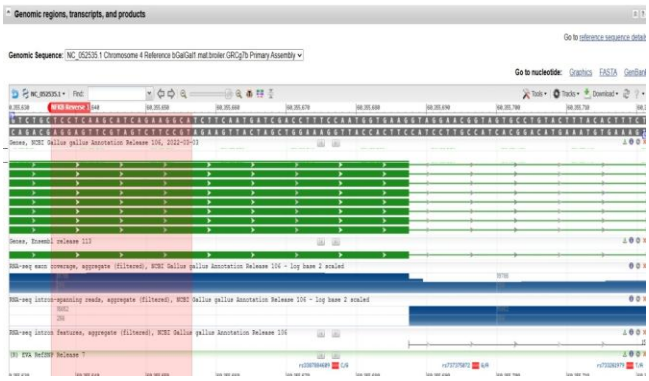

Figure 2: Nucleotide sequence of target NF-KB reverse primers designed through Gen Bank database.

Results

Brain histopathological finding

Brain histopathological lesions were observed in some infected quails, slight inflammatory cell infiltration, brain necrosis and hemorrhage, desquamation of Meninges and vascular congestion in the cerebral white matte. Additionally spongy changes, axonal sphenoid and reactive astrocyte in the cerebellum were noted, vascular hemorrhage, lymphatic perivascular cuffing with scattered inflammation, liquefactive necrosis and cerebral edema, congestion of blood vessels and gliosis were detected also, cerebral cortex necrosis and micro abscess, central nervous system also showed vascular hemorrhage, gliosis and perivascular cuffing (Figures 3 and 4). In table 2, the microscopic lesions, number of it and percent are shown. Significant greater percent was found in thickening of blood vessels 23.6% followed by hyper atrophy 22.47%, interestingly the gliosis lesion showed the low percent 10.86% while the necrosis and vasogenic edema have close percent of lesion represented by 15.73 and 14.61% respectively in contrast to the apoptotic which showed 12.73% percent lesions.

Table 2: Histopathological findings survey

Lesions	n(%)	x^2 (Sig.)
Apoptosis	34(12.73)	
Gliosis	29(10.86)	
Thickening of blood vessels	63(23.60)	21.79** (0.0006)
Necrosis	42(15.73)	
Vasogenic edema	39(14.61)	
Hyper atrophy	60(22.47)	
Total	267	

** refer to significant difference between groups at P<0.01.

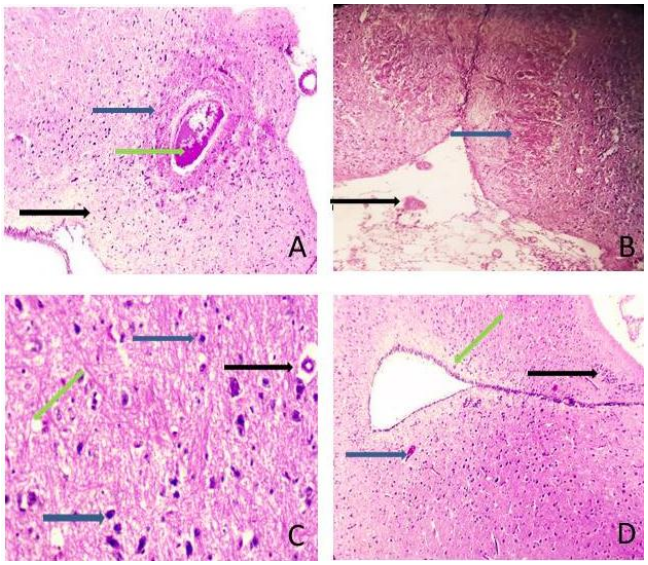

Figure 3: Microphotograph of Quails brain represent inflammatory cell infiltration (black arrow), liquefactive necrosis (blue arrow), neuronal necrosis (green arrow) A 4X, Meninges desquamation (black arrow), blood vessels (green arrow), vascular congestion (blue arrow) B 4X, spongy changes (green arrow), axonal sphenoid (black arrow), reactive astrocyte (blue arrow) C 10X, vascular hemorrhage (blue arrow), lymphatic perivascular cuffing (green arrow) scattered inflammation (black arrow) D 4X.

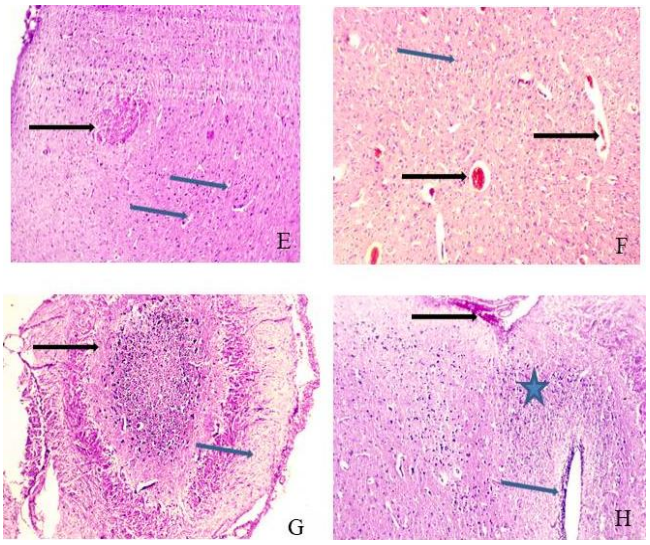

Figure 4. Microphotograph of Quails brain represent liquefactive necrosis (black arrow), cerebral edema (blue arrow) E 4X, blood vessels congestion (black arrow), gliosis (blue arrow) F 10X, cerebral micro abscess (black arrow), cerebral cortex necrosis (blue arrow) G10X, vascular congestion (black arrow), gliosis (star), perivascular cuffing (blue arrow) H10x.

IHC results

Our immunohistochemistry findings confirm prior results, all brains samples discontinuous strong glial fibrillary acidic protein expression (Figure 5).

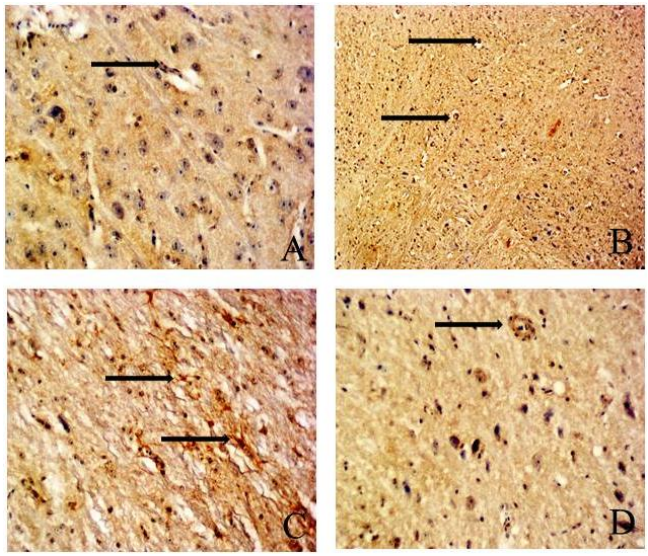

Figure 5: Quails Brain GFAP protein expression (capillary expression) A, (Glial cell expression) B, (astrocyte and spinal cord expression), (cerebral vascular expression) D, 400X.

Detection of brain finding by real time -PCR

Representative brain samples tested with real time-PCR, Figure 6 demonstrate the product size as per-step we have done to reveal the NF-kB gene expression; each color number represents diverse samples.

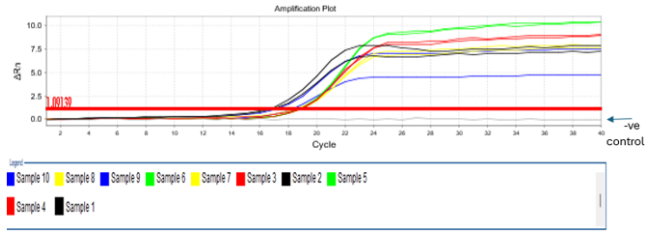

Figure 6: The One Step RT-PCR SYBR® Green Master Mix Kit was used to perform the real-time PCR amplification curves of brain samples examined for NF-kB gene expression. The normalized fluorescence signal generated by the reporter at each cycle of PCR amplification is denoted by ΔRn.

Gene expression data of our sample

Reflect variable result, as shown in table 3. sample 1and 2 revealed the highest NF-kB expression (16.75), interestingly sample 9and 10 showed the less expression in

contrast to1and 2 samples. However, the lowest expression was recorded in samples 5and 6 (18.85). the rest of samples represented by 3,4,7and 8 have close expression of NF-kB to 5and 6 while considering low expression than sample 1and 2.

Table 3: Cycle Threshold (CT) value for NF-kB gene expression in different brain samples

Sample	CT mean value
S.1	16.9
S.2	16.6
S.3	18.9
S.4	18.7
S.5	18.9
S.6	18.8
S.7	18.6
S.8	18.9
S.9	17.2
S.10	18.2

CT: Cycle Threshold of brain lesions samples.

Discussion

The diagnosis of quail nervous system lesions was based on clinical signs, histological and immunohistochemistry finding and gene expression and these were consistent with previous researcher's studies (32,33). The central nervous histopathological findings were mainly characterized by cellular adaptation and vascular changes, degeneration, necrosis and architecture alteration which may be indicating to viral infection of the brain (34,35). Although all ages of birds can be affected by avian encephalitis virus but in this paper, it was less than 3-5 weeks of age which showed the clinical lesions. Although, it is more probable that the avian encephalitis cases in this paper are due to error during the light fowl vaccination process that doesn't allow the transmission of adequate immunization to the offspring resulting in clinical signs and disease. The present paper has shown infiltration of inflammatory cell with perivascular cuffing (36,37) the inflammatory responses could be deem an expression of specific cell- immunity and these with humoral immunity ultimately lead to virus elimination from tissues as well the cuffing reaction is an indicative usually of viral infection .Necrosis of the CNS tissue caused may be varied ,in viral case infection its associated with ischemia ,anoxia or even indirectly immune mechanisms or it may be related to marked inflammatory cell infiltration, the degenerative changes represented by desquamation of meninges and axonal sphenoid usually associated with inflammatory setting particularly with macrophage cell, tract degeneration secondary to cerebral necrosis or even due to malacia (38), in this study vascular hemorrhage, blood vessels congestion and cerebral edema (39-41) who recorded vascular changes due to immunosuppression or due to viral infection. NF-KB and other concerning proteins (42) are considering transcription factors which may be related with the gene expression inside the nervous system (43) These factors are characterized by their presenting in most cells in an inactive form within the cytosol, linked to inhibitory proteins. The dissociation of inhibitory proteins is caused by many activators, such as the activation of protein kinase type C, which is an essential transport pathway for the nervous system. Various genes expressed in the nervous system, such as prodynorphin and proenkephalin are considered potential targets for this type of transcription since they guaranty elements (putative KB) (44). Exposure of ducks, geese and chickens to stress factors stimulates inflammatory factors (45). These inflammatory signs bind to the receptors and trigger intracellular inflammatory signals chain reaction resulting in increased NF-KB expression. NF-KB enhance the expression of inflammatory factors such as IL-6, IL-1and TNF- and these in turn could active the signaling pathway of (NF-KB) and promoted the inflammation responses (46,47), NF-KB is considering a key factor in the signaling pathway; it amplifies cytokines and chemokine inflammatory signal and organized the inflammation persistence throughout inflammation process. overexpression of these protein lead to inflammation responses and pathological changes in the body (48).The present work has showed intensive expression of GFAP and the expression was predominantly related to the Glial cell, astrocyte, spinal cord as well in brain cerebral (49) in the tissue of the CNS ,it well know that neurons destroyed are replaced by gliosis, the exaggerated synthesis of GFAP in the CN tissue is as a restraint to neuron devastation and plays a main role in the formation of the brain glial scar (50,51). Increased expression of this protein helps stabilize the processes of newly formed Müller cells and provides resistance against stress (52).

Conclusion

As previously mentioned, brain samples from affected quails exhibited a diverse range of histopathological findings, including inflammation, cellular adaptation, vascular changes, degeneration, necrosis, and architectural alterations. The RT-PCR analysis for the NF-KB protein revealed varying degrees of expression across all affected samples. Immunohistochemistry (IHC) for intermediate filament proteins demonstrated strong immune reactivity. Therefore, the observable lesions in our study may be influenced by viral infection, inoculation route, and variations in infection virulence. Further research is necessary to identify the specific types of viral infections and their primary causes.

Acknowledgment

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Conflict of interest

Researchers declare no collision of interest.

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التقييم الجزيئي للبروتين الخيطي المتوسط كعلامة للكشف عن الآفات المرضية النسجية لدماغ السمان

هديل باسم السبعاوي، انتصار خزعل الحمداني و أثير نبيل طه

فرع الأمراض وأمراض الدواجن، كلية الطب البيطري، جامعة الموصل، الموصل، العراق

الخلاصة

يلعب الجهاز العصبي دورا حاسما في تنظيم معظم وظائف الجسم الحيوية. قد يؤثر عامل الإجهاد أو العدوى على الجهاز العصبي المركزي بنوعية (المركزي والطرفي). يهدف العمل الحالي إلى تحديد الآفات المرضية العرضية في دماغ طائر السمان من خلال دراسات التغيرات النسجية المرضية وتحديد التفاعل المناعي باستخدام البروتين الحمضي الليفي الدبقي، وتفاعل البلمرة المتسلسل لبروتين العامل النووي كابا ب. تم الحصول على خمسة عشرة عينة من دماغ طائر السمان التي أظهرت علامات سريرية من المزارع المصابة نُقلت العينات إلى المختبر. بعد إجراء الصفة التشريحية قُطعت المقاطع بسُمك ٤-٥ مايكرومتر، وطبقت إجراءات التقطيع ثم صبغت بالصبغة الروتينية. أُجريت الكيمياء المناعية النسيجية للبروتين الحمضي الليفي الدبقي وفقا لبروتوكول الشركة، كما أُجري تفاعل البوليمير المتسلسل للعامل النووي كابا ب. أظهرت النتائج وجود التهاب وتسلل الخلايا الدبقية وتثخن الأوعية الدموية، والتكيف الخلوي، والتغيرات الوعائية، ونخر التنكس، وتغيير معالم النسيج. وجد تفاعل المناعة للبروتين الحمضي الليفي الدبقي رد فعل قوي داخل الشعيرات الدموية الدماغية، والخلايا الدبقية، والخلايا النجمية، والحبل الشوكي، ومنطقة الدماغ. اظهر التحليل الإحصائي باستخدام مربع كاي زيادة كبيرة في نسبة سماكة الأوعية الدموية وعند ٢٣,٦ وانخفاض ملحوظ في نسبة الدبق وبنسبة ١٠,٨٦%. أظهر تفاعل البلمرة المتسلسل لبروتين العامل النووي كابا ب درجة متفاوتة من التعبير بين جميع العينات المأخوذة، وكان أعلى تعبير في العينة ١ و٢ (١٦,٧٥)، ومن المثير للاهتمام أن العينة ٩ و١٠ أظهرت أقل تعبير. ومع ذلك، تم تسجيل أقل تعبير في العينات ٥ و٦ (١٨,٨٥). بينما بقية العينات والمتمثلة بالعينات ٣ و٤ و٧ و٨ لها تعبير وثيق. ونستنتج أنه على الرغم من أن السمان مقاوم للظروف البيئية إلا أنه عرضة لعوامل الإجهاد أو أي نوع من أنواع الإصابة والتي تؤثر على كفاءة الجهاز العصبي المركزي.

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