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RESEARCH ARTICLE

Antibiotic Abuse Induced Histopathological and Neurobehavioral Disorders in Mice

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ARTICLE HISTORY

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Abstract: ***Introduction*:** Antibiotic abuse is a common phenomenon in Egypt as medications are prescribed without supervision. It is suggested that the excess use of antibiotics modifies the gut microbiota and plays a role in the development of neurological and psychiatric disorders.

***Objective*:** The aim of the present study was to use bulb-c mice as models for curam (amoxicillin /clavulanic acid) abuse compared to the locally acting neomycin model, then restoring the probiotic balance to look at the possible effects on the animal brains**.**

***Methods*:** The results showed early excitable brains demonstrated by S100b immunohistochemistry in both cortexes and hippocampuses of neomycin-treated mice. Staining with PAS stain showed no suggested neurodegenerative changes. Treatment with probiotics improved the S100b immunohistochemistry profile of the curam group partially but failed to overcome the neuroinflammatory reaction detected by hematoxylin and eosin stain. Curam was possibly blamed for the systemic effects.

***Results*:** The neurobehavioral tests showed delayed impairment in the open field test for the curam group and impaired new object recognition for the neomycin group. These tests were applied by video recording. The neurobehavioral decline developed 14 days after the end of the 3-week antibiotic course. Unfortunately, curam abuse induced animal fatalities.

***Conclusion*:** Antibiotic abuse has a neurotoxic effect that works by both local and more prominent systemic mechanisms. It can be said that antibiotic abuse is a cofactor behind the rise of neuropsychiatric diseases in Egypt.

Keywords: Antibiotic abuse, microbiota, curam, neomycin, S100b, PAS stain, gut-brain axis.

1. INTRODUCTION

Antibiotic abuse is a major health hazard in the middle-east. Unfortunately, parents can obtain antibiotics from the pharmacy without control. Depending on faulty concepts, they think that every case of upper respiratory tract infection should be managed by antibiotics even without consultation of the physician, making antibiotic abuse a commonly accepted cultural trend [1]. People think that they are the best choices and use them on the basis of self-medication. Moreover, when people feel better, they don't follow the complete course of the antibiotics [2].

An Egyptian study examining antibiotic abuse in Cairo showed that most common cases exposed to unnecessary antibiotic courses were children suffering from chest infections. The most common categories used were penicillin derivatives and those of the cephalosporin group [3]. Similar reports showed an excess use of antibiotics in the management of urinary tract infection in China [4]. Another study conducted in the kingdom of Saudi Arabia demonstrated that Amoxicillin-clavulanic acid was the most commonly used antibiotic in the private sector to manage dental cases in Al-Madinah Al-Munawarah [5].

Recently, a study recorded cases of psychosis related to the management of urinary tract infection by multiple antibiotics like fluoroquinolones, penicillins, and trimethoprim-sulfamethoxazole. The authors demonstrated that psychosis developed during and after the end of the antibiotic course. The mechanism behind the acute and remote delusional cases has not been explained [6].

Long-term antibiotic therapy for tuberculosis has been reported to induce clear neurotoxicity including cognitive disorders and delusional thinking. The mechanisms behind neurotoxicity need further research [7].

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The gut-brain axis has been a target of extensive research recently. The GIT (Gastro-intestinal tract) has up to 10^{15} bacteria that exceed the number of cells in humans. This huge population is considered to be the second genome that modifies the mood and alters brain development. It was well-known for more than a century that gastric fistula with altered intestinal microbiology population was associated with mood disruption. The immune reaction against the GIT flora influences cytokine machinery with a strong impact on brain function and development [8, 9].

The balance of the microbial population has been considered as an independent biological factor that affects the hypothalamic-pituitary axis. Antibiotic abuse caused the animals to show anxiety-related disorders. This could be related to the activity of the microbial population secreting neuropeptides and altering the immune activity that modulates the brain activity. The cytokines secreted by the immune system find their way to the brain by the lymph or the leaky blood-brain barrier [10-13].

The recent wide-scale research considered the gut as a second brain with the products of bacterial metabolism changing the manufacturing process of serotonin, the key neurotransmitter related to mood and implicated in the pathogenesis of depression [14].

The probiotic formulation consists of combined Lactobacillus helveticus R0052 and Bifidobacterium longum R0175 expressed anxiolytic features in animal models and non-complaining human subjects. A study showed improved cognitive functions with probiotic therapy in candidates suffering from abdominal pain [15].

Liu and his group [16] concluded that there was increasing locomotor activity and decreased anxiety-like behaviour in specific pathogen-free (SPF) mice after daily administration of newly isolated *Lactobacillus plantarum* PS128 (PS128). The improved behavioural tests were associated with elevated bio amines in the striatum that may explain the anti-anxiety properties of the probiotics and the possible role in the improvement of the motor scoring.

The aim of the current study was to evaluate the neurological effects of curam and neomycin courses on bulb-c mice as models for antibiotic abuse. Neomycin was chosen as the locally acting control to be compared with curam, the popular antibiotic used for upper respiratory tract infection in Egypt. The animals were expected to have a neurobehavioral and histological impairment. Probiotic therapy was applied to overcome the expected pathology.

2. MATERIALS AND METHODS

This work was an experimental study which was Approved by the Ethical Committee of Faculty of Medicine, Mansoura University. It investigated the effect of antibiotic abuse on neurobehavioral tests in mice related to intestinal dysbiosis. The study was performed in the Medical Experimental Research Center (MERC).

2.1. Materials

2.1.1. Animals

Eighteen male Balb-c mice aged seven weeks with weights between 20-25 gram, were obtained from the animal house of the Medical Experimental Research Center (MERC), Faculty of Medicine, Mansoura University, Mansoura, Egypt.

The animals were housed in a specific room with a suitable temperature (22±2 °C), good lightening (12 hours light /dark cycles) and good aeration. The animals were fed a standard laboratory diet and tap water and treated groups were separated from each other to avoid cross-contamination.

2.1.2. Chemicals

1- Curam (Amoxicillin + Clavulanic acid): Oral suspension 312.5mg from Sandoz Company.

2- Neomycin 500 mg: As a locally acting agent, aminoglycoside antibiotic from Memphis Company for pharmacy and chemical industry, Egypt.

3- Mood Probiotics: By innovixLabs, Canada, two Strains of *Lactobacillus helveticus* Rosell-52ND and Bifidobacterium longum Rosell-175 were used.

4- Sodium thiopental 1000 mg, phosphate buffered solution (PBS) and paraformaldehyde (PFA): They were obtained from Medical Experimental Research Center (MERC), Faculty of Medicine, Mansoura University, Mansoura, Egypt.

5- Meyer's Hematoxylin and eosin stain: were obtained from the Pathology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt.

6- Periodic Acid Schiff (PAS) Stain: was obtained from the Pathology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt.

7- Immunohistochemistry (IHC): Antibodies for mAb S100b (NBP1-956) from NOVUS, conc 0.1ml rabbit were applied.

8- Serum blocking solution: 10% non-immune serum, hydrogen peroxide and methanol were used.

2.1.3. Instruments

1. ANY-box® (Stoelting Company, USA): It was used to assess the neurobehavioral changes. ANY-box is a multi-configuration behaviour apparatus designed to automate a range of standard behavioural tests. ANY-box consists of two components; an ANY-box base and core. ANY-box base includes a camera to track the animals. To expose mice to different tests, different enclosures are used. Each enclosure fits the ANY-box base. As many as eight enclosures for different tests can be used to automate any of the ANY-box behavioural tests.

2. Standard light microscopy (Olympus® model CX31RTSF, Tokyo, Japan) attached to the digital camera (Olympus® model E-420, China).

3. All needles and syringes for injections, scalpels, test tubes and glass slides were obtained from MERC.

2.1.4. Software

A video tracking system designed to automate testing in behavioral experiments was used for the analysis of neurobehavioral tests.

2.2. Methods

2.2.1. Experimental Setup

2.2.1.1. Animals and Housing

Male mice, aged 7 weeks-old, were provided with standard laboratory diet and water. After one week adaptation period, 18 male Balb-c mice weighing approximately 20 -25 gm were randomly distributed into three groups; each group had 6 mice (N6) and tail marking was done.

2.2.1.2. First Phase (Antibiotic Administration)

Group 1 -Neomycin group- (N6) This group received antibiotics in the form of neomycin (350 mg/l) to deplete primarily Gram-negative and some Gram-positive bacteria. Neomycin was given by dose 0.025 mg/g once daily by oral therapy for three weeks [17].

Group 2 -Curam group- (N6) This group received antibiotics in the form of Curam oral suspension 312.5 mg. Curam (312.5) was given by dose 0.01 mg/g once daily by oral therapy for three weeks [18].

Group 3 -the control group- (N6) This group was the placebo (PL). This group received the 0.5% methylcellulose vehicle from days 1 to 21.

2.2.1.3. Second Phase (Probiotics Administration)

Group 1 (Neomycin group): Received nothing.

Group 2 (Curam group): Received daily probiotics by oral therapy. Probiotics were given by dose 0.1 mg/ml once daily by oral therapy for two weeks.

Group (PL) 3: Received the 0.5% methylcellulose vehicle.

2.2.2. Assessment Methods

Assessment of the effect of dysbiosis caused by antibiotic was done by neurobehavioral tests and histopathological examination of the brain.

2.2.2.1. Neurobehavioral Tests

The locomotor activity of the mice was evaluated at the end of the first and second phase of the study by the use of ANY-box tests.

Two behavioral tests (*i.e.* two enclosures were used) were adapted from ANY-box; dark and light test and open field test.

2.2.2.1.1. Open-field Test

Open-field test apparatus has been prepared to measure the neurobehavioral scores in mice for research purposes. This apparatus is constructed of a clear acrylic plastic box 40cm x 40cm x 35cm (width, length, height) and fits on ANY-box base. Two perpendicular lines were drawn on the floor with a marker and were visible through the clear wall. These lines divided the floor into four equal quadrants: northeast, northwest, southeast, and south-west. The tests were performed in a room that was completely isolated from external noise.

At the center of the apparatus, the animal was located individually for observation for two minutes. Ethyl alcohol 70 % was used to clean the open-field floor between each mouse. Each mouse trial was recorded to be analyzed later, using a camera that was placed on the top of the apparatus. The neurobehavioral function was assessed for each animal for the number of mid-zone crosses [19].

The open field test aimed to asses mainly anxiety through the number of midzone crosses. Anxious mice tend to avoid the central zone. The test was also applied to evaluate locomotor activity.

2.2.2.1.2. Dark and Light Test

The device used for the light/dark transition test consisted of an acrylic box 40cm x 40cm x 35cm (width, length, height) fitted onto ANY-box base that was divided into two sections of equal size by a partition with a door. Mice were left to move freely between the two chambers for 2 minutes. Each animal was left individually at the dark portion of the device and observed for two minutes. The Apparatus floor was cleaned between each mouse using 70 % ethyl alcohol. Each mouse trial was recorded for later analysis, using a camera positioned above the apparatus. The neurobehavioral functions could be assessed by using the following parameters: The distance traveled in each chamber, the total number of transitions, the time spent in each chamber, and the latency to enter the light chamber [20].

We used the test to asses anxiety through the time passed in the Lightroom. Anxious mice tend to spend more time in a dark room.

2.2.2.1.3. New Object Recognition Test

The object recognition test is now among the most commonly used behavioral tests for mice. A mouse is presented with new objects and allowed to explore it. The amount of time taken to explore the new object provided an index of recognition memory [21].

The time taken to explore new objects was used to measure cognitive function. Anxious mice were expected to spend less time exploring new objects.

2.2.2.2. Histopathological Evaluation

At the end of the second phase, under deep anesthesia with thiopental (100 mg/kg, intraperitoneal), the mice were perfused through the aorta with 50 mL of 10 mM Phosphate-Buffered Saline (PBS), followed by 150 mL of 4% paraformaldehyde. After perfusion, each brain was rapidly dissected and fixed for 2 days with 10% paraformaldehyde. The brain pieces were processed into paraffin blocks and then cut by microtome at 4-5 micron on glass slides [22].

Brain sections were dehydrated, mounted on the slides and examined with standard Olympus® light microscope (model CX31RTSF). Pictures were captured by a digital camera (Olympus® model E-420).

2.2.3. Techniques of Used Stains

Slides obtained from the brain were stained by hematoxylin and eosin stain. Periodic acid-Schiff (PAS) stain was applied for the same brain sections. The immunohistochem-

Table 1. The comparison between antibiotic (Neomycin and Curam) receiving groups in comparison to control regarding the neurobehavioral tests. The antibiotic groups showed no statistically significant changes from the control.

Neurobehavioral Tests	Control Number of Animals =6	Neomycin Number of Animals 6	Curam Number of Animals 6
Open field	5.50 ± 1.05	4.33 ± 1.03	4.80 ± .84
Dark and light test	51.67 ± 3.98	48.83 ± 4.58	51.00 ± 1.58
New object recognition test	47.17 ± 5.71	52.83 ± 1.47	51.80 ± 2.59

**p*-value is significant if ≤0.05.
Data expressed as mean± SEM.

istry used S100b (S100 Calcium Binding Protein B) to stain the brain cortexes and hippocampuses.

2.2.4. Hematoxylin and Eosin Stain

Paraffin sections were dewaxed in xylol and hydrated through descending grades of alcohol to distilled water. Then, sections were put in Harris's hematoxylin for 5 minutes and washed in running tap water for 5 minutes. The next stage was differentiation of the tissue sections in 1% acid alcohol (1% HCl in 70% alcohol) for 5-10 seconds.

Sections were washed in running tap water again until they became blue. Then, staining in 1% eosin for 10 minutes was carried out, followed by washing with distilled water. Finally, the sections were dehydrated through ascending grades of alcohol, cleared in xylol and mounted in Canada balsam [23].

2.2.5. PAS Stain (Periodic Acid Schiff)

The slides were deparaffinized and hydrated with water. The slides were oxidized in 0.5% periodic acid solution for 5 minutes. The slides were rinsed with distilled water, stained with Coleman's feulgen solution for 15 mins and then washed in running tap water for 10 mins and counterstained in Harris hematoxylin for 1min. The next step was to wash in running tap water for 5 minutes.

The slides were differentiated in 1% acid alcohol for 30 seconds. After that, the slides were washed in running tap water for 1 minute. The next phase was bluing in 0.2% ammonia water or saturated lithium carbonate solution for 30 seconds to 1 minute, dehydrated and cover slipped using a synthetic mounting medium [24].

2.2.6. Immunohistochemistry by S100 (S100 calcium-binding protein B)

Slides underwent deparaffinization with xylene and were dehydrated in descending grades of alcohol (100% - 90%-70%) each for 5 min. The slides were washed in a buffer solution for 10 min. Blocking of endogenous peroxidase using the 3% hydrogen peroxide in methanol for 5 min was applied. The samples were then washed in buffer for 5 min. The next step was epitope retrieval by boiling in a pressure cooker with 0.01 M HIER Citrate Buffer pH 6.5.

The next step was washing in buffer solution for 5 min, then the slides were incubated with proteinase K 0.04% for 5 min. After that, the slides were washed with PBS for 5 minutes and incubated with the anti-human monoclonal antibodies with a dilution of 1:100 in a humid chamber for 1 hour.

The slides were washed in PBS 3 times for 2 min each, and then 2 drops of the secondary antibody were added and incubated for 10 min. The next step was to wash in PBS 3 times for 2 min each. 2 drops of strept-avidin-biotin were added for 30 min at the room temperature and washed in PBS 3 times for 2 min each. 2 drops of Diaminobenzidine (DAB) were added and used as the chromogen for 10 min. Again the slides washed in PBS 3 times for 2 min each and 2 drops of Mayer's hematoxylin counterstain were added for 1-3 min. The slides were put in the buffer for 30 seconds, then washed in distilled water, dehydrated using ascending grades of alcohol and cleared in xylene for 5 min before mounting [25].

2.2.7. Image Analysis

Captured images were analyzed using Image J (32-bit) software for windows.

2.2.8. Statistical Analysis

The collected data were coded, processed and analyzed using the SPSS (Statistical Package for Social Sciences) version 15 for Windows® (SPSS Inc, Chicago, IL, USA). Qualitative data were presented as number and percent. Comparison between groups was done by Chi-Square test. Quantitative data were presented as mean ± SD. A paired t-test was used for comparison within groups. A student t-test was used to compare between two groups. $P < 0.05$ was considered to be statistically significant.

3. RESULTS

3.1. Neurobehavioral Tests

- ***Phase (1)***

Table **1** illustrates the difference between the neomycin group and the control group with respect to numbers of mid-zone cross in the open field test, time spent in the light-zone in the dark and light test, and time spent exploring new objects in new object recognition test. There were no statistically significant differences in the neomycin group in comparison to the control group.

Table 2. The comparison between both antibiotic groups namely curam and neomycin, two weeks after the end of antibiotic phase one course. The first group was the control receiving no medication. The neomycin group received nothing for spontaneous recovery. The curam group received probiotic for 2 weeks after the end of phase one. The probiotic corrected group showed depressed activity in the open field test. The neomycin group showed depressed activity in the new object test.

Neurobehavioral Tests	Control = 6	Curam (N=4) (Probiotic)	Neomycin (N=6)
Open field	5.67 ± 1.03	4.25 ± .50*	4.83 ± .75
Dark and light test	50.17 ± 2.48	53.25 ± 1.71	49.83 ± 3.13
New object recognition test	45.83 ± 4.54	48.00 ± 3.37	42.00 ± 2.83*

**p*-value is significant if ≤0.05.
Data expressed as mean± SEM.

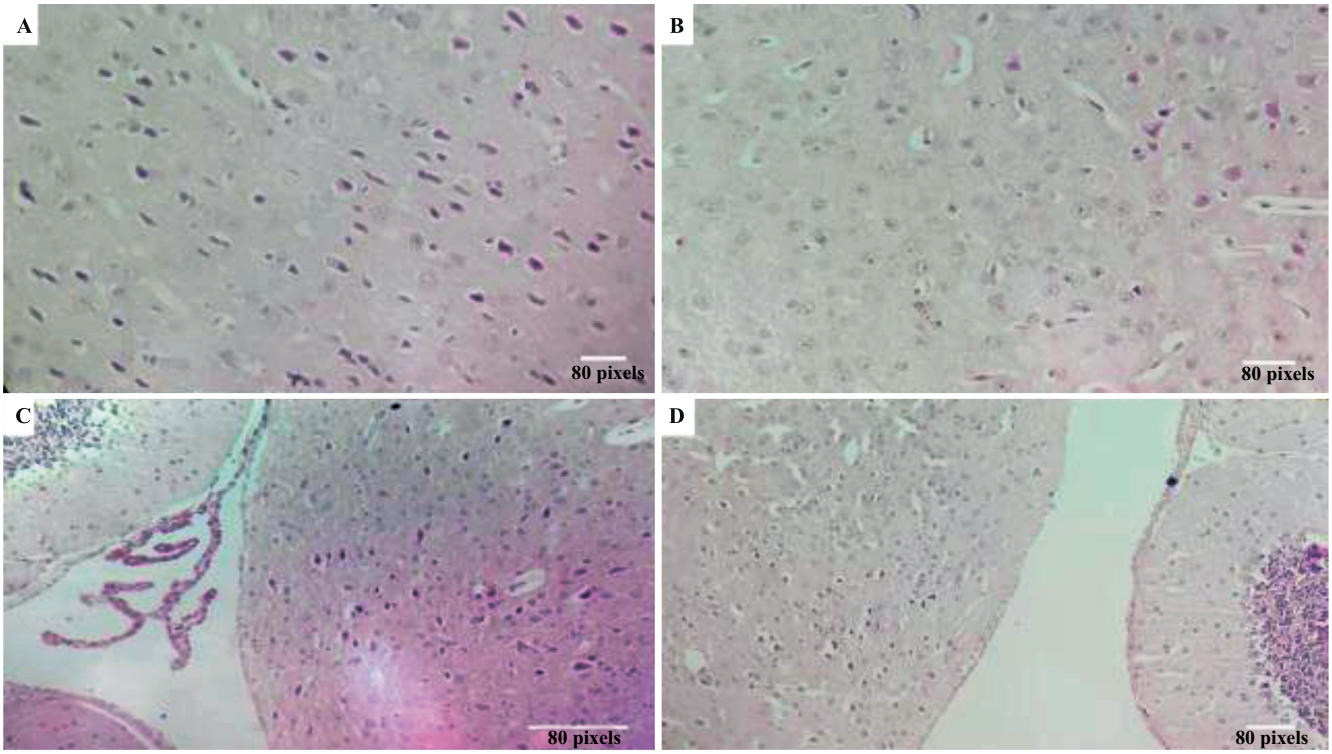

Fig. (1). A) Microscopic picture with H&E of the control group showing brain cortex with normal histology and no evidence of inflammation, degeneration or apoptosis x400. **B**) Microscopic picture with H&E of neomycin group showing brain cortex with normal histology as the control one x400. **C**) Microscopic picture with H&E of the control group showing brain hippocampus with normal histology with no evidence of inflammation, degeneration or apoptosis x200. **D**) Microscopic picture with H&E of the neomycin group showing brain hippocampus with normal histology as the control one.

- ***Phase (2)***

Table **2** illustrates the difference between the control, the curam/probiotic group and the neomycin group after cessation of the antibiotic courses for two weeks with respect to numbers of mid-zone crosses in the open field test, time spent in the light zone in the dark and light test, and time spent exploring new objects in new object recognition test. The curam/probiotic group showed depressed activity in the open field test. The neomycin group expressed lowered new object finding capability.

3.2. Histopathology

After the end of the experiment, the brain and the hippocampus of each animal were fixed and examined by H&E. The brain samples were immune-stained with S100b and then were stained with PAS stain. The neomycin group did not show an inflammatory reaction, neither degeneration nor apoptosis, by H&E examination of either cortex or hippocampus slides (Fig. **1A**-**D**). However, S100b activity was increased in the samples taken from the cortex and hippocampus (Fig. **2A**-**D**).

The samples taken from the curam/probiotic group showed inflammation in the cortex and the hippocampus sections by H&E examination (Fig. **3A**, **B**). Staining with S100b showed a variable response. Some animals showed completely recovered S100 activity in cortical slides (Fig. **3C**) and hippocampal slides (Fig. **3D**). Others showed partial recovery (negative staining of S100b) in cortical slides in

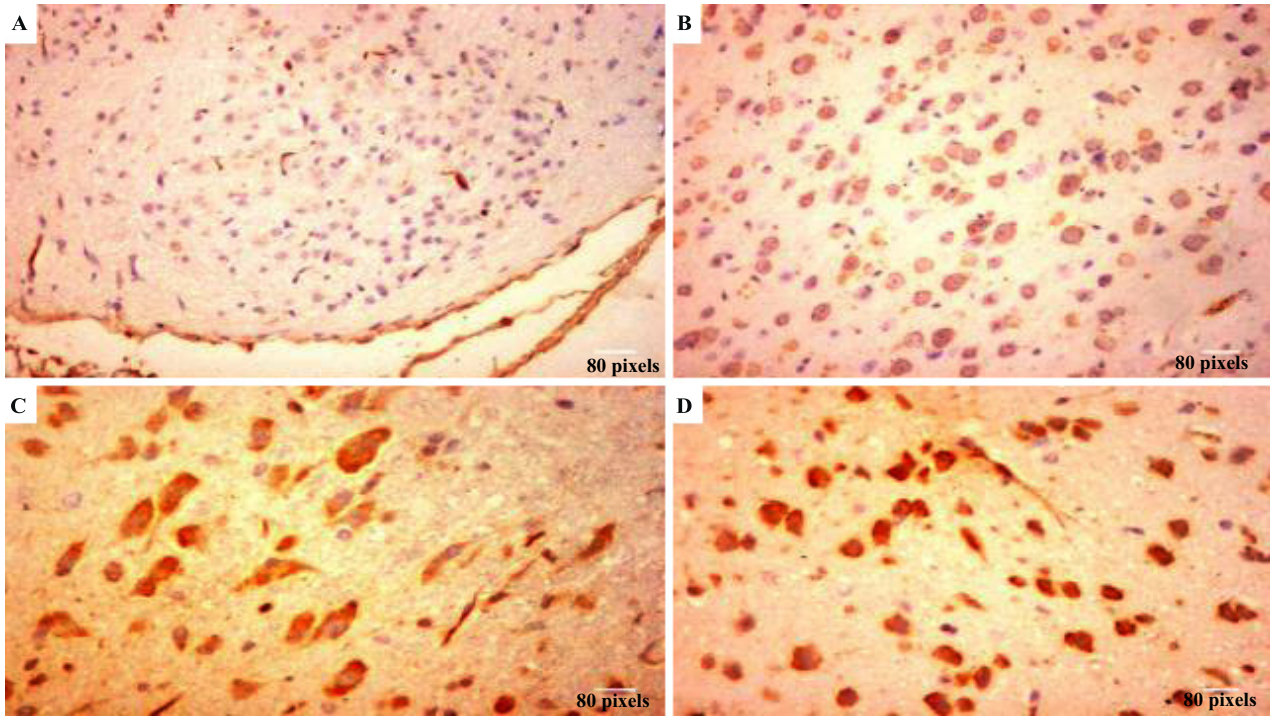

Fig. (2). A) Microscopic picture of brain cortex of control group stained with S100 immune stain showing negativity of neurocytes for S100b but the covering pia matter membrane was positive taking the brown color x200. **B**) Microscopic picture of brain cortex of the neomycin group stained with S100 immune stain showing brown granular cytoplasmic positivity of neurocytes for S100 x200. **C**) Microscopic picture of brain hippocampus of the control group showing moderate intensity of brown granular cytoplasmic positivity of some of the neurocytes for S100 immune stain x400 **D**) Microscopic picture of brain hippocampus of the neomycin group expressing strong intensity of brown granular cytoplasmic positivity of all neurocytes for S100 immune stain x400. *(The color version of the figure is available in the electronic copy of the article).*

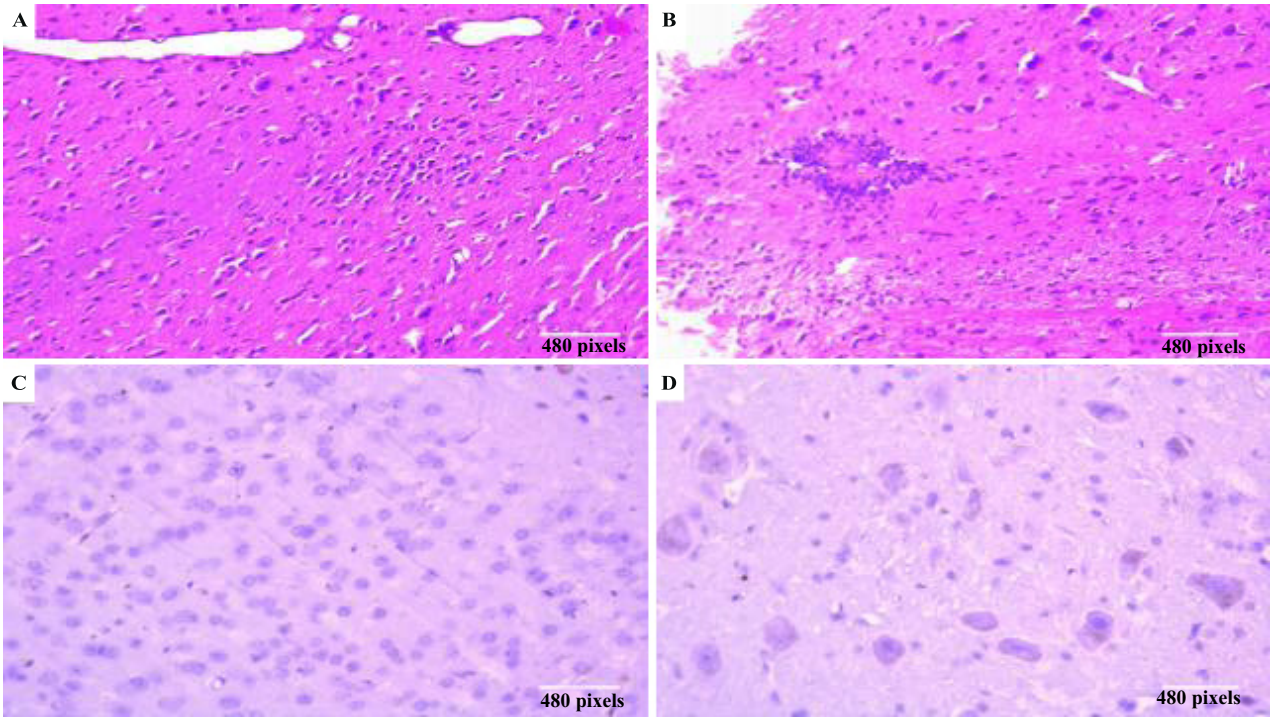

Fig. (3). A) Microscopic picture of brain cortex of the curam+probiotic group demonstrating infiltration of the brain tissue with aggregates of inflammatory lymphocytes x200. **B**) Microscopic picture of brain hippocampus of the curam+probiotic group expressing inflammatory granuloma x200. **C**) Negativity of brain cortex neurocytes of the curam+probiotic group (mice 2&4) for S100 immune stain x400 **D**) With weak granular cytoplasmic positivity of brain hippocampus neurocytes of the curam+probiotic group (mice 2&4) for S100 immune stain x400.

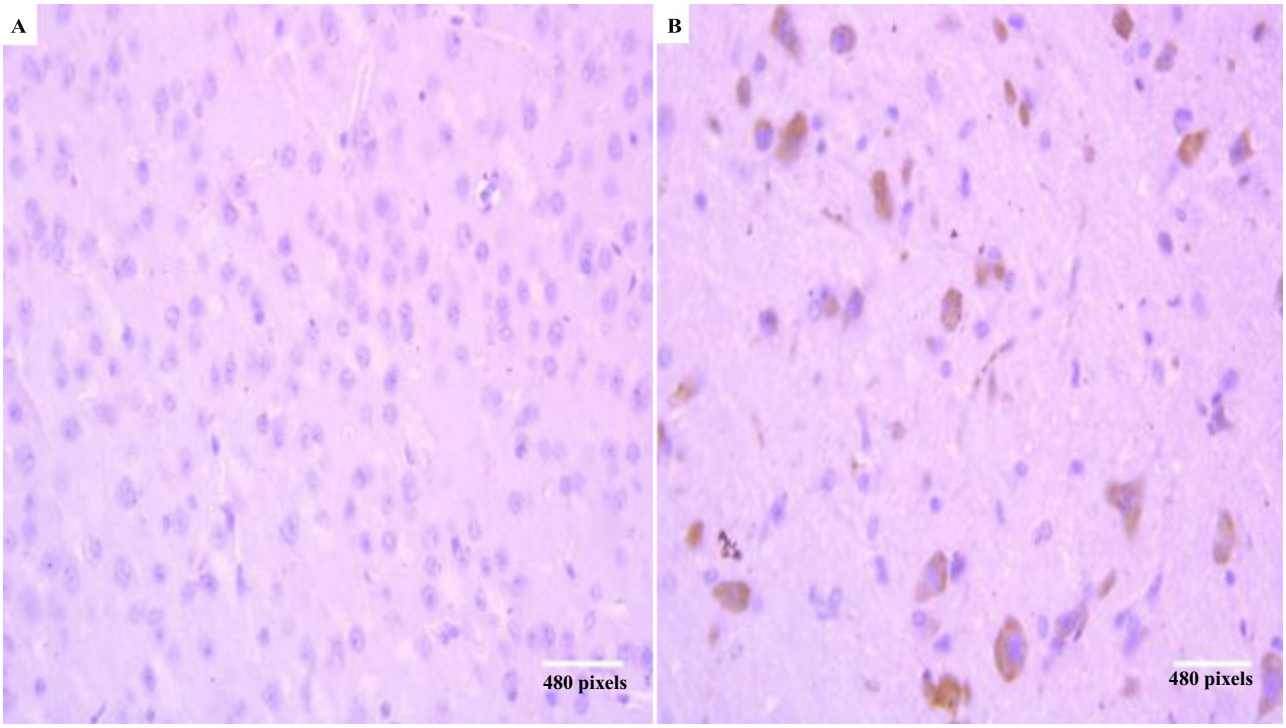

Fig. (4). A) Negativity of brain cortex neurocytes of the curam+probiotic group (mouse 6) for S100 immune stain x400. **B**) Moderate positivity of brain hippocampus neurocytes of the curam+probiotic group are demonstrated (mouse 6) for S100 immune stain x400.

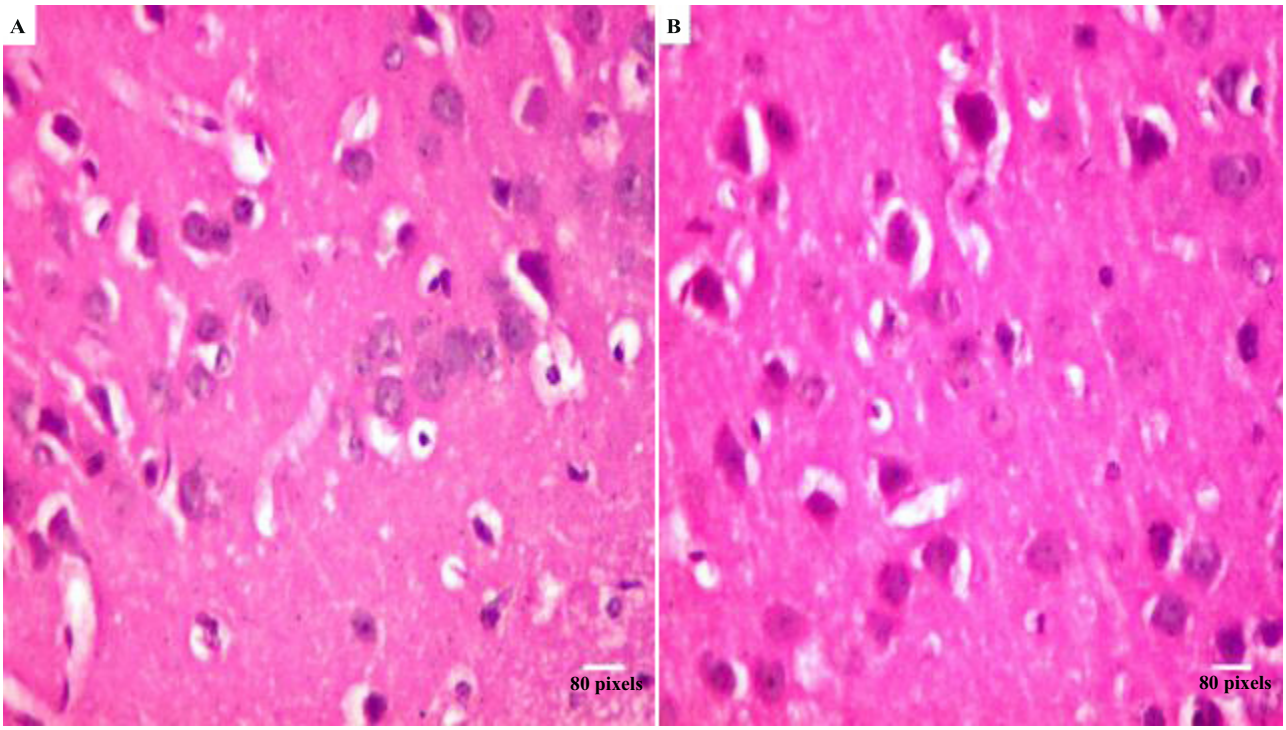

Fig. (5). A) Negativity of brain cortex neurocytes of neomycin group for PAS stain. **B**) Shows negativity of brain hippocampus neurocytes of neomycin group for PAS stain.

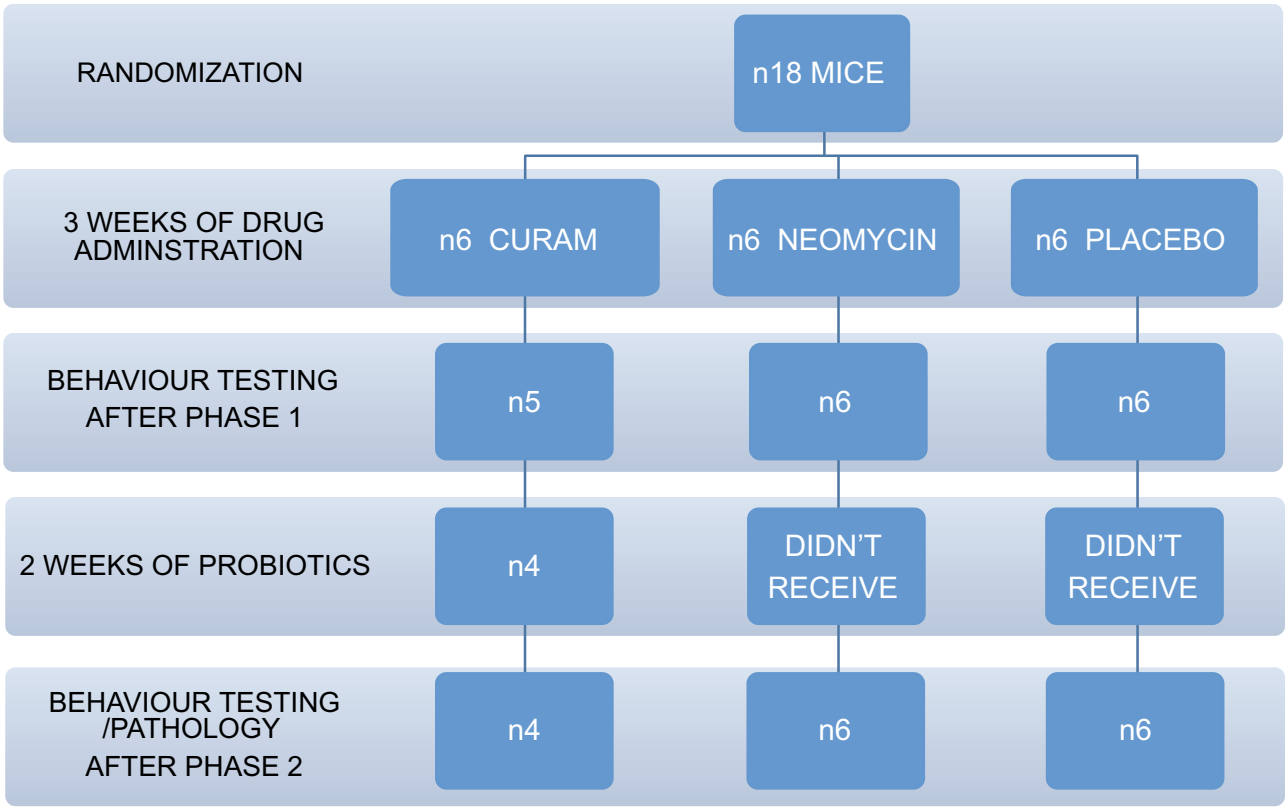

Fig. (6). A flow chart of mice numbers over the duration of the experiment. The curam group expressed 2 animal fatalities out of 6. One animal died the first phase and the second one died two weeks later in phase 2. No fatality was detected in both the control and the neomycin groups.

contrast to hippocampal slides that showed moderate staining (Fig. **4A**, **B**). The neomycin group showed no evidence of degeneration or apoptosis of the neurocytes. Fig. (**5A**, **B**) showed negative PAS staining in the cortical and the hippocampal slides of the neomycin group.

3.3. Animal Fatality

During the course of the study, two animals died in the curam group. One mouse died in phase (1) and the other one died in phase (2) (Fig. **6**).

4. DISCUSSION

The current experimental study was held in the Mansoura Medical Experimental Research Center to study the effects of antibiotic abuse and probiotics on mice neurobehavioral responses. The study was conducted on BALB-C mice. The animals were subjected to a course of the popular antibiotic, curam, used for the treatment of chest infection affecting children in Egypt. The local community has already misused classic penicillin for years and moved to curam combination to overcome resistance. In this study, the course of curam was split into 2 phases with both negative control and neomycin groups representing locally acting antibiotic.

The current work tracked 3 behavior tests including the open field test, the dark and night test and the new object finding the test. The first one is a marker for locomotor activity, the second one can be used to assess anxiety and the third one explains the memory and cognitive function. The level of anxiety affects all the tests or one of them. Unfortunately, by the end of the course, one curam group animal died.

In the second phase of the experiment, probiotics were given for two weeks to study their protective effects on the group of mice exposed to curam. Another animal died in the curam group despite probiotic therapy. All groups were tested for the same neurobehavioral tests in the first phase. For the anxiety-like behavior and brain pathology, the neomycin group was left to recover without any more interference. The animals were sacrificed and the brain cortexes and hippocampus were examined by H&E. Immune stain with S100b was applied to show calcium activity as a shadow of neuronal excitability. PAS stain was applied to stain mucin and starch that may be a marker for neurodegeneration. The histopathological results demonstrated neuro-inflammation in the curam group suggesting systemic effects in contrast to the neomycin one. Different changes in the pathogen population between the two groups may be behind the inflammatory findings. Further research is needed to explain why neuroinflammation occurs as a systemic complication of curam abuse. The S100 immune stain showed excess stain in the neurocytes especially in the hippocampus region of the neomycin group. Such results were associated with the impaired object finding score. Both findings strongly suggest the role of intestinal flora modification and possibly depletion in the pathogenesis of anxiety and depression (Fig. **6**). Probiotic therapy to the curam group partially improved the S100 results with a less intense reaction but did not solve the neu-

roinflammation findings. This neuropathology was associated with impaired locomotion behavioral tests. More studies are needed to confirm their biological mechanisms. Curam is theoretically exerting a combined systemic and local effect on the gut-brain axis by modifying the bacterial demography. PAS stain showed no significant changes between the curam, neomycin or control groups. The impaired neurobehavioral findings may suggest a memory disease model but no neurodegenerative findings were demonstrated by PAS staining. It is suggested that disrupting the gut-brain axis by antibiotic abuse is not the best model for neurodegenerative diseases like Alzheimer's.

These results coincide with the results of Bercik who created an animal model for infective colitis and treated the animals with the probiotic, *Bifidobacterium longum* NCC3001 [17]. Interestingly, his results showed improvement of the anxiety level without recovery of histological inflammatory complications. The cell line results indicated decreased excitation of the enteric neural cells on exposure to the probiotic therapy. However, the current research demonstrated evident impaired neurobehavioral tests in the late phase two weeks after the antibiotic course on the wild-type animal model.

Bruce-Keller and his group concluded that obese depressed patients suffered more depression as a result of altered microbiota in the intestine related to a fatty diet. The mouse model exposed to high fatty diet expressed anxiety. They explained neuroinflammation findings as a result of a high blood level of endotoxins. Bruce-Keller and his group found increased expression of calcium binding adapting molecule 1, which supports the S100b results of our current work [26]. Interestingly, Bruce-Keller demonstrated the increased activity of Toll-like receptors 2 and 4 as a potential clue to neuroinflammation. The current work expressed the neuropathology in a remote way and suggested that antibiotic abuse in the Egyptian community may be responsible for neuropsychiatric disorders decades later. Recent work found that a chronic unpredictable mild stress of Sprague-Dawley rats created a model of a depressed animal showing increased S100B protein expression in the rat hippocampus [27].

The gut-brain axis is bi-directional with many proposed mechanisms. The first one is vagal related stimulation. The second theory is the modulation of the immune system. The third hypothesis is the gut hormone signaling and a fourth mechanism is a pathogenic approach to the central nervous system [28, 29]. Stable microbiota has the ability to produce neurotransmitters like GABA the receptors for which are a well-known anxiolytic drug target [30]. The hypothesis is that the excess antibiotic use works as a choice factor for harmful pathogens that use different mechanisms to induce neuropathology.

Atli and his group [31] studied the potential toxicity of amoxicillin on animals. He found neurotoxicity effects in the form of convulsion attacks, depression, and changes in the behavior, which supported the current study findings. Atli and his group explained his findings by the decrease of brain serotonin and increased glutamate. The animals showed increased oxidative stress markers and it seems that intense oxidative stress may explain the inflammatory brain reaction in the current study.

Different lactobacillus and bifidobacteria species have been shown to modulate depression and stress-related behaviors in animal models. The probiotic bacterium *Lactobacillus rhamnosus* can directly increase the single and multiunit firing rate of the mesenteric nerve bundle. It can decrease stress-induced corticosterone and anxiety/depression in mice.

Lots of studies noticed the importance of probiotic therapy in the treatment of anxiety and depression but we think that oral probiotic supplementation is a promising but adjuvant medication, as it partially improves the histological scoring [32-35].

Limitations of this study include lacking sequencing of the bacterial DNA complex in the animal's guts. Also, the study did not check the probiotic effects on the neomycin group. The study focused on curam as it is one of the most abused antibiotics in the Egyptian community. Neomycin is not a commonly abused antibiotic like the ampicillin and cephalosporin families. The study recorded fatalities in animals treated with curam therapy. These adverse effects can be related to the pathology of other organs.

CONCLUSION

The current study examined animal models for antibiotic abuse. Excess use of antibiotics like Amoxicillin Clavulanic combination induced neurotoxic effects demonstrated by neurobehavioral tests and histopathology. Neomycin-induced hippocampal excitability was detected by S100b that reflected calcium binding protein activity. However, the systemic inflammatory reaction to curam was not resolved by probiotic therapy. The neurobehavioral tests showed potential impaired memory, cognition and motor function in a remote way. PAS stain showed no evidence of neurodegenerative changes. The results also put the spot on the inter-individual variation in the bacterial environment in the intestine. Probiotic therapy is expected to be an adjuvant medication of anxiety and depression disorders.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work was an experimental study which was approved by the Ethical Committee of Faculty of Medicine, Mansoura University, Egypt.

HUMAN AND ANIMAL RIGHTS

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author (A.M.N.H.) upon reasonable request.

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None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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