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Article:

Nile tilapia (*Oreochromis niloticus*) response and salt mitigation effect post 5 hours transportation stress

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Abstract

In aquaculture facilities, routine processes such as live transportation has been done on the Nile tilapia juvenile to be reared and for breeding purposes. Live transportation activates the stress response compromising welfare of fish In the current study, a 5hour transport stress model was used to investigate how ***Oreochromis niloticus*** (*O. niloticus)*, especially the skin, responds to transportation stress and to detect the salt's mitigation effect on such stress. A total of 150 ***Oreochromis niloticus*** (53±3gm) were divided into three equal groups. The first was a non-transported control group (P1), the second was the (PT2) group, in which the fish were transported in a salt free water and the third was the (PT3) group, where a 5 g/L of salt was added to the water during transportation. The study results revealed that water pH and the dissolved oxygen levels decreased non-significantly ,while the total ammonia level increased significantly in the PT2 and PT3 groups compared with the control group. Survival rates of *O. niloticus* were 90 % and 96 % in PT2 and PT3 groups, respectively. In addition to , the bacterial colony forming units (CFU) in the liver and spleen of the P1 group was negligible($0.17 \times 10^3 g^{-1}$), however it was highly and progressively raised in the PT2 group and slightly elevated in PT3 group compared with the P1 group. Moreover , the serum glucose level at 0 hour post transportation was significantly higher in the PT2 group while no significant difference was recorded in the PT3 group comparing to the control group. However, at 12 h and 24hours serum glucose significantly decreased in both PT2 and PT3 groups, but no significant differences between the two groups were found. The histopathological study of the skin revealed marked differences in the PT2, PT3 groups compared with the control group. This study indicated that transportation had stressful effects on *O. niloticus*, and Nacl helped in mitigating the transport stress effects.

Keywords: Nile tilapia, *O. niloticus*, salt, stress, Transportation

Introduction

Nile tilapia (*Oreochromis niloticus*, *O. niloticus*) was introduced in developing countries to meet local protein needs **[1].** Many aquaculture operations including transportation of fish from one facility to another or during restocking practices are unavoidable and inevitable event in aquaculture **[2,3],** and fish transportation is an extremely stressful **[4]**. Stressful events increase circulating cortisol levels and trigger gluconeogenesis and increase energy supply needed to cope with the stress **[5]**. Stress suppresses the immune responses in stressed fish **[6,7]**. Transportation events could lead to stress to fish, including overcrowding, deterioration of water quality, change of temperature and dissolved oxygen **[8]**. Transportation stress led to ammonia accumulation, hydromineral imbalance and mortality in different fish species, such as the winter flounder (*Pseudopleuronectes americanus)* **[9]**, Cobia (*Rachycentron canadum)* **[10]**, and red porgy (*Pagrus pagrus*) **[11]**. The skin of fish is an important first barrier against pathogen entry and it is equipped with a mucosal immune system known as SALT **[12,13]**. Disruption of skin barrier homeostasis and

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dysregulation of skin commensals as a result of stress can potentially explain the increased disease susceptibility overgrowth in stressed individuals of Rainbow trout **[14].** The practice of adding salt to water for transportation of freshwater fish as an attempt to mitigate transport stress is questionable **[15,4]** and contradicted by other studies **[3,16].** This conflict is attributed to differences among fish species that have different response capacities **[17]**. In the current study, *O. niloticus* was selected for our investigation because of its economic importance as it is the most cultivated fish species in Egypt and one of the most cultivated fish species in the world. In addition, Nile tilapia is diseases and environment resistant, has a relatively short cultivation period, and has a relatively low production cost **[1]**. we investigated *O. niloticus* resistance to live transport and the stress mitigation effect of Nacl following a 5-hour transport model. We compared 3 different groups, the first was a non-transported group (P1), the second was a transported group without salt (PT2) and the third was the transported group in water containing 5g/L Nacl (PT3) as a stress mitigator.

Material and methods

Ethical Considerations

All experimental protocols and procedures were conducted by the ethical committee guidelines of the Faculty of Veterinary Medicine, Sohag University. The ethical approval number is Soh. Un. Vet/00016 M1.

Experimental design

A total of 150 *Oreochromis niloticus* were obtained from a private Wadi Samhod tilapia farm in New Valley Governorate. Fish were subdivided into three groups (50 fish/group), the first group was used as a non-transported control group (P1), the second group was transported in water without salt (PT2) and the third group was transported in water containing 5g/L Nacl (PT3). Transport water was obtained from the farm's pond. Each fish group was transported in a separate tank (180Liter capacity) containing 100Literof water. The stocking density during transportation was 26.5g/L. Fish were transported for 5hours without stopping, sedative drugs, and with continuous aeration to the wet lab of Fish Diseases and Management Department, Faculty of Veterinary Medicine, Sohag University. Temperature, dissolved oxygen, pH, and total ammonia were determined before and after transportation.

Sampling

Blood and skin samples were collected before transportation (control group, P1), and at 0 hr., 12 hr. and 24 hr. Five fish from each experimental group were sampled at each determined time point and were anesthetized with MS-222(150 mgL^{-1}) **[18].** Blood samples were collected after anesthesia from the caudal blood vessels with non-heparinized syringes and added in1.5 ml Eppendorf tubes. The blood samples were left to clot at room temperature, then centrifuged at 1200 xg (relative centrifugal force) for 10 min. The sera were collected gently and stored at -80°C until analysis. Immediately after blood sampling, skin sample was collected then preserved in 10% formalin for histopathology examination.

Water quality parameters

The water dissolved oxygen, pH, and total ammonia were determined before and after transportation using portable probes (Hach Co., Colorado, USA) and a photometer (Palintest Co., Tyne, UK).

Survival rate

After transportation was finished, the fish containers were put into the wet lab for observation and recording the survival rate at 0, 12, and 24hours post-transportation. Total survival rate was calculated by subtracting the number of the dead fish from the initial number of fish at the end of the transport stimulation experiment.

Glucose level measurement

Serum glucose was determined using a calorimetric enzymatic test from a commercial kit glucose liquicolor (Human mbH, Germany) . According to the kit procedure, absorbance readings were taken using a spectrophotometer (HITACHI–U–2001; Japan) with a 500 nm wavelength.

Bacterial translocation

Liver and spleen tissue samples from each fish were collected under sterile conditions, weighted, and then homogenized. After homogenization, the spleen and liver were separately suspended in 400 µL of sterile phosphate buffer saline (PBS), and 20 µL of the resulting solution was plated onto Tryptic Soy Agar (TSA) plates. Plates were incubated in an incubator for 18-24hours, and CFU numbers were counted. The result of the viable bacterial count was expressed as colony-forming units (CFU) per ml of sample **[19].**

Histopathology

Skin samples were fixed in 10% paraformaldehyde for paraffin embedding. Five µm-thick paraffin sections were stained with hematoxylin-eosin as well as with alcian blue/periodic acid-Schiff (AB/PAS stain) at two different pH values (1 and 2.5) in order to reveal the chemical composition of mucosal secretion and visualize different mucins as explained elsewhere **[20].** The skin goblet cells were counted under a microscope and scored as blue or magenta.

Histopathologic Scoring

Each sample was assigned a score based on tissue histopathological examination **[21]**. The samples were scored semi-quantitative, with assessment based on the visual field inspection of a minimum of 10 sections from each group. Photographs were taken at a magnification of 40 X, and tissue alterations were scored according to set criteria: 1, 2, 3, and 4 (absent, mild, moderate, and severe, respectively) **[22]**, Skin and muscular tissue sections were analyzed for the following alterations: epidermal cell thickness, vacuolation, inflammatory cellular infiltration, dermal vascular congestion, inflammation, hypodermal cystic dilatation and mucoid degeneration, inflammation, melanocytes distribution and degree of deposition, muscular fibers irregularity, degeneration, hemorrhage, and inflammation. The analyses were performed by two researchers by recording the nature and extent of the lesion and its frequency of occurrence in randomly selected sites in the tissue **[23].**

Statistical analysis

Data of all measurements from experimental groups were stated as mean ± standard deviation (SD), and they were estimated by the use of GraphPad Prism Version 5 (San Diego, California, USA).The data was analyzed using one-way ANOVA with Tukey's post-hoc multiple comparison tests; and two-way ANOVA with Bonferroni post-test to compare replicate means by row, the statistical significance was considered at $P<0.05$ **[24,25]**.

Results

Water quality parameters

The results of our study revealed that the temperature, pH, dissolved oxygen (DO), and total ammonia before transportation were 30 ºC, 7.89, 7.23 $mgL^{-1}$and 0.20 mgL^{-1} ,respectively. At 0h post-transportation; temperature was 33.4 ºC in the experiment tanks, the pH values in the PT2 and PT3 fish groups were 7.18, 7.10 and DO levels were 6.62 and 6.41mgL^{-1} ,while total ammonia levels were 0.33 and 0.59 mgL^{-1}. At 12h post-transportation; temperature was 26.6 ºC in the experiment tanks, the pH values in the P2 and PT3 groups were 7.60 and 7.90, DO levels were 7.01 and 6.91 $gL^{-1}$and total ammonia levels were 0.29 and 0.34 mgL^{-1}. At 24h post-transportation; temperature was 28.4 ºC in the experiment tanks, the pH values in the PT2 and PT3 fish groups were 7.50 and 7.80, DO levels were 7.14, 7.00 mgL^{-1} and total ammonia levels were 0.22 and 0.26mgL^{-1} ,respectively in the PT2 and PT3 fish groups **Table 1**.

Survival rate

O. niloticus in PT2 group which transported in water without salt recorded a 90% survival rate, while the fish in PT3 group which transported in water containing 5g/L salt recorded a 96%survival rate. The survival rate was recorded within the first 24hr post-transportation **Table 2**.

Bacterial translocation

The liver and spleen of *O. niloticus* in the control group (P1) contained a negligible number of bacterial CFU (0.17 x 10^3). The total bacterial count in the liver and spleen at 0, 12, and 24 h post-transportation in PT2 group greatly increased to 16, 29.4 and 36.7 folds, respectively. Meanwhile in the PT3 group, it moderately increased to 1.14, 6.02 and 6.9 folds at 0, 12, and 24 h post-transportation, respectively when compared with the control group **Table 3**.

Table (1): The temperature, pH, DO and total ammonia values at different experiment times.

Fish groups	pH			
	Time			
	Before	**0 time**	**12hr**	**24hr**
PT1	7.89	-	-	-
PT2	-	7.18	7.60	7.50
PT3	-	7.10	7.90	7.80
	DO –mgL^{-1}			
PT1	7.23	-	-	-
PT2	-	6.62	7.01	7.14
PT3	-	6.41	6.91	7.00
	Ammonia - mgL^{-1}			
PT1	0.20	-	-	-
PT2	-	0.38	0.26	0.22
PT3	-	0.59	0.28	0.26
	Water Temperature			
	26.5 – 33.5 ºC (30 ± 4.95ºC)			

Table (2): Number and percentage of survival rate of all examined *O. niloticus* at 0, 12, 24 h. post transportation

Time	Survival rate		
Fish group	**P1**	**PT2**	**PT3**
0 h	-	1	-
12 h	-	1	1
24 h	-	3	1
Cumulative number	-	5	2
Percentage	-	10%	4 %

Table (3): Average number of bacterial CFU gm-1 in liver and spleen of all examined *O. niloticus* before, at 0, 12 and 24 h. post transportation

	Groups					
	P1		**PT2**		**PT3**	
	Number of bacterial colonies forming units /gm					
	No.x10^3					
	Liver	**Spleen**	**Liver**	**Spleen**	**Liver**	**Spleen**
BT	0.18	0.15	-	-	-	-
	0.17x103					
0 hr. PT	-	-	2.87	2.40	0.16	0.18
			16 folds		**1.14 folds**	
12 hr. PT	-	-	4.5	5.10	0.89	1.10
			29.4 folds		**6.02 folds**	
24 hr. PT	-	-	6.4	5.70	0.99	1.30
			36.7 folds		**6.9 folds**	

BT : Before transportation, PT: post transport

Glucose

The glucose level of *O. niloticus* in control group (P1) was 226.1mgdl^{-1}. At 0 h post transportation, the serum glucose level was significantly increased in the PT2 group recording 302.33mgdl^{-1} and persisted near to the basal level with non-significance decrease in the PT3 group reporting 193.8 mgdl^{-1} in comparison to the P1 control group, and the glucose levels were significantly decreased in the PT3 fish group compared to the PT2 fish group. At 12 h post transportation; the glucose significantly decreased to 184.40 and 137.6 mgL^{-1} in the PT2 and PT3 groups in comparison to the P1 control group, the glucose level was significantly dropped in the PT3 group in comparison with the PT2 fish group. At 24 h post transportation; glucose level was significantly decreased to 157.16 mgL^{-1}in the PT2 group and 169.7 mgL^{-1} in the PT3 fish group compared to the P1 control group, its levels were non significantly decreased in the PT3 fish group in comparison to the PT2 fish group **Table 4**. and **Figure 1.**

Histopathology

The histopathological study of the *O. niloticus* skin revealed marked differences between the PT2 and PT3 groups and the P1 group. The total lesion scores revealed that the skin sections of the PT3 group manifested the lowest histopathological alterations (no inflammatory signs at 0h and mild inflammatory signs at 12 h and 24h), while the skin sections of the PT2 fish group showed mild inflammatory signs at 0 h and moderate inflammatory signs at 12 and 24h post transportation. Skin tissue sections from *O. niloticus* transported in water without salt showed moderate inflammatory reactions in the form of melanocytes aggregation, atrophied and loosened muscle bundles, vacuolated hypodermis, and the absence of goblet cells, reflecting the skin response to the stressful conditions of transportation. Adding 5g/L of salt to the transportation water mitigated the stressful conditions of transportation in the PT3 group and improved the skin histopathology in the form of normal epidermis layer with goblet cells, dermis with tight fibrous connective tissue, melanocytes filled with melanin pigment and normal hypodermis. Summing up, the histomorphometry graph showed semi-quantitative measurements of total lesion scores recorded in skin sections among the experimental fish groups, the total lesion scores revealed that the skin sections of the PT3 group manifested the lowest histopathological alterations, no inflammatory signs at 0h and slight inflammatory signs at 12 and 24h post-transportation, while the skin sections of the PT2 fish group appeared mild inflammatory signs at 0 h and moderate inflammatory signs at 12 and 24h post transportation **Figure 2,3.**

Table (4) Glucose levels at before, 0, 12, 24 h. of transportation. expressed as means ± standard deviations.

Fish group	Time	Sample No.	Glucose level(mg/dl)
P1 (Control group)	**Before transport**	1	231.80
		2	226.60
		3	219.90
		Mean± SD	226.10±5.97
PT2 (without Nacl)	**0 h post transport**	1	309.20
		2	301
		3	296.80
		Mean± SD	302.33±6.31***
PT3 (5g/L Nacl)		1	182.43
		2	193.80
		3	205.17
		Mean± SD	193.8±11.37 ns
PT2	**12 h post transport**	1	176
		2	186
		3	191.20
		Mean± SD	184.40±7.73*
PT3		1	140.20
		2	131.80
		3	140.80
		Mean± SD	137.60±5.03***
PT2	**24 h post transport**	1	166.40
		2	164.68
		3	140.40
		Mean± SD	157.16±14.54***
PT3		1	160.8
		2	173.2
		3	175
		Mean± SD	169.7±7.73***

Significant differences vs. the control group (P1) are marked by different asterisks, all through one-way ANOVA with Tukey's post hoc test: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). ns means non- significant compared with control (P1) group.

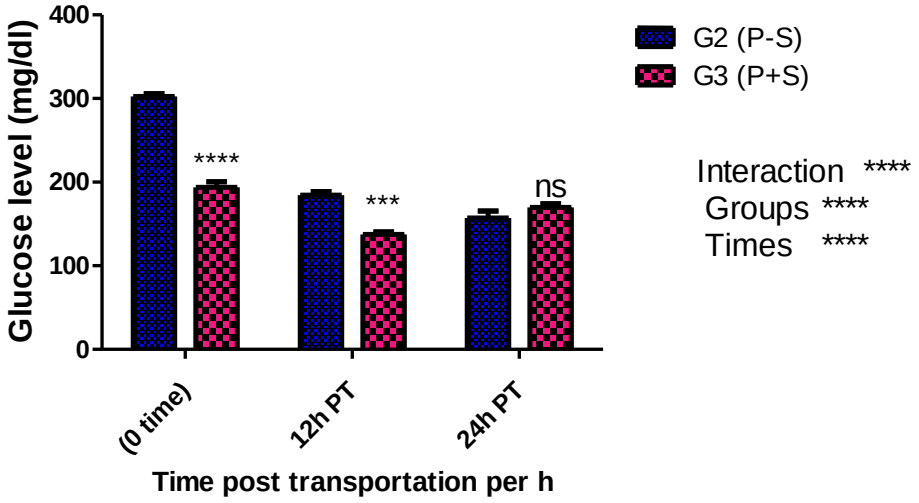

Figure (1): Glucose measurements in all experimental groups and the interaction between group-time factors. Data are expressed as means ± standard deviations. Significant differences vs. the control group are marked by different asterisks through two-way ANOVA with Bonferroni post -test to compare replicate means by row: * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001). ns means non- significant compared with PT2 (p-S) group.

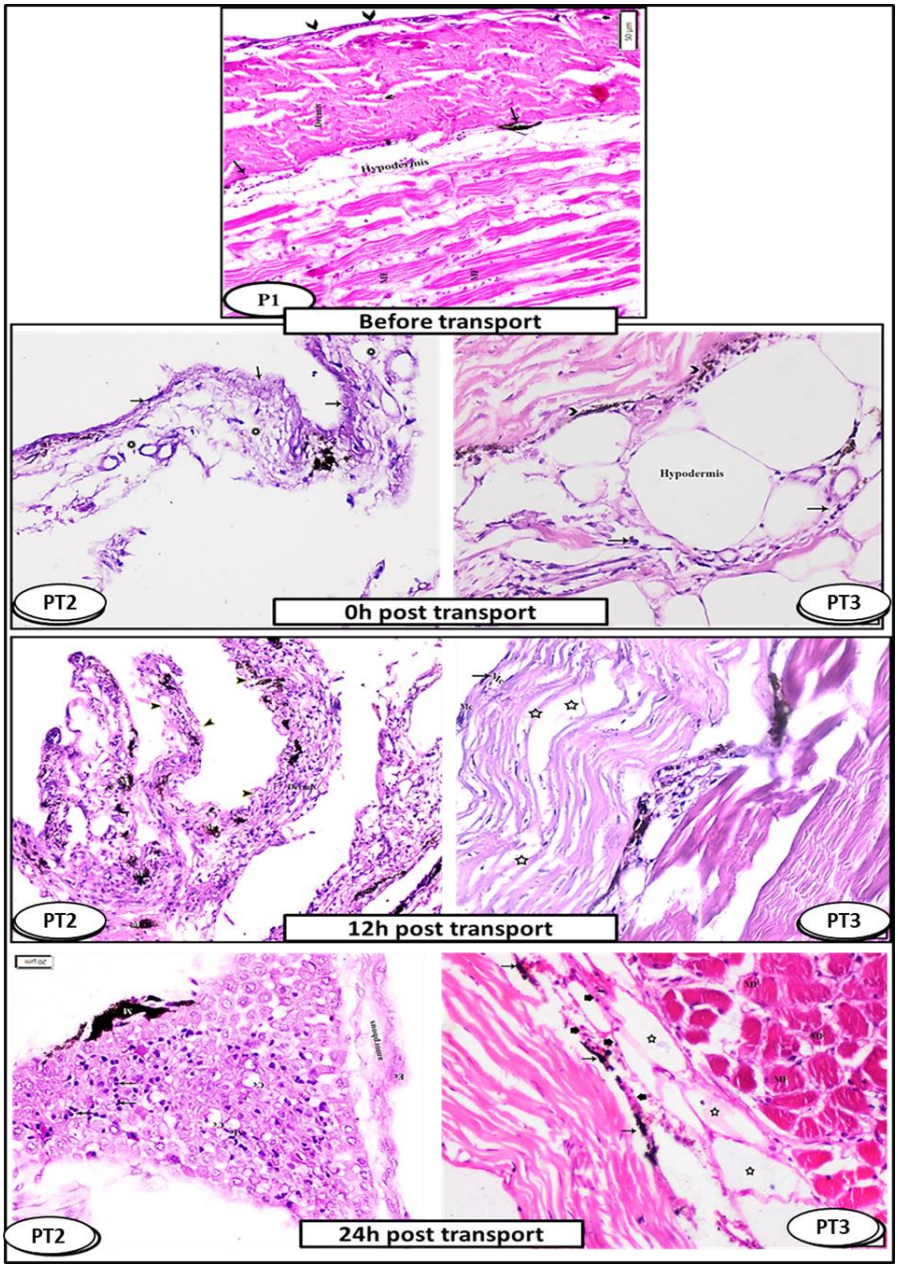

Figure (2): Skin tissue sections from *O. niloticus* at different times and from different groups. **Before transport: P1 group (control)**; show normal skin structure including normal epidermis (multilayered flattened epithelial cells (arrowheads) with goblet cells), dermis (fibrous connective tissue, melanocytes (arrows)), and underlying muscle fibers (MF) (bar=50mμ). **At 0 h post transport: PT2 group (without salt);** show normal regular collagenous bundles of dermal layer (stratum compactum), melanocytes (zigzag arrow), normal hypodermis, normal muscle fibers (MF) (bar=50mμ). **PT3 group (with 5g/L salt);** show normal epidermis (arrowheads) and dermis (bar=100mμ). **At 12h post transport: P2;** show melanocytes aggregation (arrowhead) under dermis, atrophied and irregular muscle bundles (arrows), which left vacuolation in its sheath (stars) and no goblet cells detected. **PT3;** show thin flattened epithelial cells with goblet cells (arrowheads), the dermis is composed mainly of tight fibrous connective tissue, comprises a thin upper layer (loose connective tissue) and a thick dense layer (stratum compactum), melanocytes filled with melanin pigment (arrows), normal hypodermis and more or less muscle fibers (MF). **At 24h post transport: PT2;** show excess melanin (arrows), vacuolated hypodermis. Atrophied, loosened muscles fibers (M), (bar = 50μm). **PT3;** show melanocytes filled with melanin pigment normally distributed (arrows), cystically dilated hypodermis (stars), mononuclear inflammatory cellular infiltration (arrowheads) and loosely degenerated muscle fibers with peripheral located nucleus (MF), (bar =50 μm).

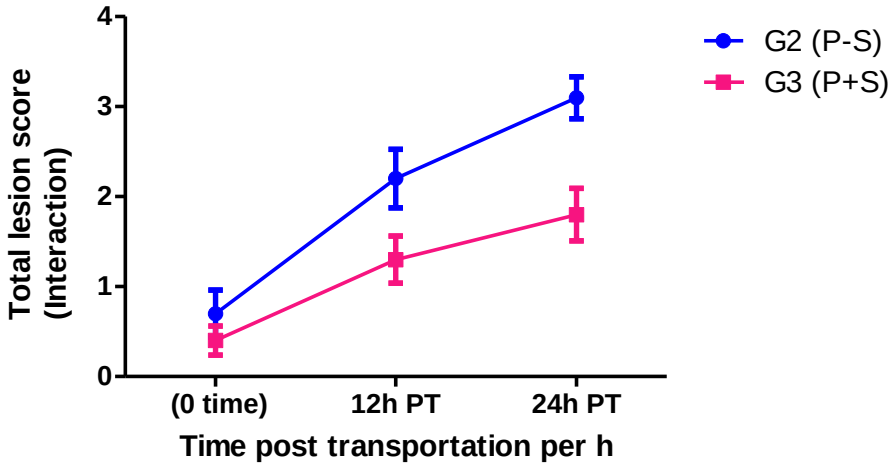

Figure (3): Histomorphometry graph showed semiquantitative measurements of total lesion scores recorded in skin sections among the experimental groups and the interaction between group- time factors. Data are expressed as means ± standard deviations. Significant differences vs. the control group are marked by different asterisks through two-way ANOVA with Bonferroni post -test to compare replicate means by row: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Discussion

Many aquaculture operations, including the transportation of fish from one facility to another or during restocking practices could not be avoidable. The immune response is stated to be suppressed in stressed fish **[6,7].** The previous studies on the immunity response of fish to stress have focused on systemic parameters such as blood cell counts and serum innate immune factors, but the role of skin immune barriers has been overlooked. In the current study, we will elicit *O. niloticus* resistance to live transport and the stress mitigation effect of salt. We used a 5-hour transport stress model to investigate how *O. niloticus,* particularly the skin, responds to stress. Regarding the water quality, transportation stress markedly increases the fish metabolic rate, which increases oxygen consumption as well as the excretion of ammonia and carbon dioxide **[26].** The results of our study showed negligible and non-significant changes in water pH before and after transportation, and this may be due to the high water alkalinity that prevents the pH fluctuation. Also, dissolved oxygen slightly and non-significantly decreased during transportation, which may be attributed to continuous aeration compensating for the consumed dissolved oxygen. On the other hand, the total ammonia significantly increased in both transported *O. niloticus* groups, and the increase was higher in the fish group transported in water containing salt indicating that transportation stress increased the fish metabolic rate and ammonia excretion, and the addition of salt may enhance the *O. niloticus* vitality and metabolic rate during transportation leading to more ammonia excretion. In consistent with our study, transportation of shark (*pangasianodon hypophthalmus)* in water with 4 g/L salt for 3 h significantly increased the water ammonia without any changes in the dissolved oxygen **[27].** Also, Pacman catfish (*Lophiosilurus alexandri)* were transported for 3 h in water with 4-8 g/L of salt reported an increase in ammonia level **[3].** On the other hand, our findings contradicted the findings of another study, which concluded that when common carp were transported for 5 hours in water containing 3 gL-1 salt, dissolved oxygen increased significantly, and water ammonia decreased **[15]**; this conflict may be due to the differences in fish species and stocking density, transportation method, basal water quality, and fish metabolic state. The survival rate of *O. niloticus* investigation after transportation revealed that the fish groups transported in water with salt showed a higher survival rate than those transported in water without salt and the fish group transported in water containing 5g/L salt showed the highest survival rate (96%). This study revealed that the addition of Nacl salt particularly 5g/L to the transport water supported the *O. niloticus* life and enhanced the survival rate, and this may be attributed to the stress mitigation and antimicrobial effects of salt, as well as the salt enhancement effect on fish hydromineral balance. A prior study on Nile tilapia found that their transportation with 4 and 8 gL-1 salt increased their life span and survival rate **[28],** which was consistent with our findings. Bacterial translocation is a common indicator of fish physical barrier breakdown and can lead to inflammation**[29,30].** The mucus continuously forms anti-biofilm and prevent the pathogens from invading again **[31,32]**. The results of this

study showed a negligible number of the bacterial CFU ($0.17 \times 10^3 g^{-1}$) in the P1 group, which revealed good *O. niloticus* health and good farm pond conditions. The transported fish groups showed a progressive increase in the bacterial CFU count in the liver and spleen at 0, 12, and 24 h post transportation. The fish group that had been transported in water containing salt appeared to have the lowest increase in the bacterial CFU count matching the fish group transported in water without salt, this may be partially due to the marked mucous secretion control and the up regulation of skin antimicrobial peptides β D 1 & 2 during transportation with their antibacterial properties **[33,34]** rendering the skin environment unfavorable for the skin microorganisms blooming and bacterial translocation to the internal organs. The serum glucose levels at 0 h post-transport were significantly higher in the PT2 group, and no significant difference was recorded in the PT3 group compared with the control group. These findings were consistent with a study conducted on *O. niloticus*, which found highly elevated glucose levels post transportation **[35].** Also, high glucose levels were reported in *Arapaima gigas* **[36]** and *Ancistrus triradiatus* **[37]** after transportation**.** This hyperglycemia may be attributed to the cortisol elevation, which triggers gluconeogenesis raise blood glucose to combat stress **[17]**.The lower glucose values in the *O. niloticus* transported PT3 group compared to the PT2 group referred to the mitigation effect of salt during transportation as a result of the reduction in the salinity difference between the fish body and the transportation water. The serum glucose levels at 12 h and 24h post transportation were significantly lower in the transported fish groups compared with the control group, and there is no significant difference between both transported groups. This may be referred to as a decrease in the glycogenolysis process as a result of the decreased cortisol level during these periods. The histopathological study revealed marked differences in PT2 and PT3 *O. niloticus* groups to the skin of the control P1 group. The total lesion scores revealed that the skin sections of the PT3 group manifested the lowest histopathological alterations (no inflammatory signs at 0h and mild inflammatory signs at 12 and 24h), while the skin sections of the PT2 fish group appeared with mild inflammatory signs at 0 h and moderate inflammatory signs at 12 and 24h post-transportation. Skin tissue sections from *O. niloticus* of the PT2 group showed a moderate inflammatory reaction in the form of melanocytes aggregation under dermis, atrophied and loosened muscle bundles, vacuolated hypodermis, and absence of goblet cells. In the PT3 group, the skin sections showed, to some extent, a normal epidermis layer with goblet cells, dermis with tight fibrous connective tissue, melanocytes filled with melanin pigment, and a normal hypodermis. It is known that transport stress leads to changes in the goblet cells number and amounts of mucus production in the skin of teleost fish **[38,39]**. The results of this study suggest that adding Nacl to the transport water may act as a retardant for the release of mucus from goblet cells in response to transport stress, which is supported by a similar study that found the goblet cell numbers decreased in eels transported from freshwater to saltwater **[40]**.

Conclusion

O. niloticus transported in water without salt was exposed to higher transportation stress and bacterial infection that appeared in the form of a low survival rate and moderate skin inflammatory reactions, in addition to the highest total bacterial CFU count. This study sheds light on the beneficial effects of 5g/L sodium chloride's addition to the transportation water of *O. niloticus* through improving fish physiology and mucosal health, reducing bacterial activity and invasion, preserving skin surface integrity, and its physical barrier function.

Authors contributions

The work was equally distributed between authors. All authors have read and approved the final version of the manuscript.

Conflict of interest

There is no conflict of interest.

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