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To elucidate the neuroprotective effect of omega-3 fatty acid supplementation in acute spinal cord injury subjects: A prospective longitudinal cohort study

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ABSTRACT

Background: Spinal cord injury (SCI) is an extremely debilitating disorder. Efforts in augmenting neurological recovery and quality of life (QoL) spinal cord independence measure (SCIM) following SCI are being studied the world over. Omega 3 fatty acid is important polyunsaturated fatty acids with some roles in normal cellular metabolism they have Anti-inflammatory and antioxidant properties have a neuroprotective effect and have a role in axon regeneration. **Aims and Objectives:** The purpose of this study was to assess the effect of omega-3 fatty acid supplements on the recovery of neurological function in SCI patients. **Materials and Methods:** In our prospective longitudinal cohort study, we enrolled 26 SCI subjects and followed for 6 months, cases were selected according to selection criteria given by the American Spinal Injury Association (ASIA) Scale guidelines. Each of the enrolled study subjects was exposed to detailed personal history that was recorded in pre-designed pro forma, subjects were divided into two groups, i.e., Group 1 comprising subjects who were on conventional treatment, Group 2 comprising subjects who were on omega-3 fatty acid supplementation (465 mg docosahexaenoic acid + 75 mg eicosapentaenoic acid) daily. Clinical assessment of ASIA scores and ASIA impairment scale and QoL (SCIM) was done at the time of admission and at all visits. The analytical tool used to assess the omega-3 fatty acid level through serum sample is Elisa. Data were analyzed by SPSS version 26 SPSS Inc Chicago IL USA. **Result:** In our study, the omega-3 fatty acid-supplemented group showed gradual increasing trends in subsequent follow-ups, and the difference was statistically significant ($P<0.0001$*), whereas receiver-operating characteristic analysis of omega-3 levels between cases (at different follow-ups) and controls, a significant cutoff value of 3.90 with a sensitivity of 92.31% and specificity of 84.62% was observed. Similarly, in comparison to Group I, omega-supplemented group had improved QoL as SCIM scores raised through time in Group II in comparison to Group I. **Conclusion:** Omega-3 fatty acid, a neutraceutical, helps in facilitating neurological and functional recovery in acute SCI in comparison to subjects on conventional treatment.

Key words: Acute spinal cord injury; Spinal cord independence measure; Asia impairment scale; Nutraceutical; Axonal regeneration; Omega-3 fatty acid; Enzyme-linked immunosorbent assay; Randomized controlled trial; Polyunsaturated fatty acids; Docosahexaenoic acid; Intervention

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INTRODUCTION

Traumatic spinal cord injury (SCI) has complete or partial cord damage that can lead to loss of motor, sensory, and autonomic functions.[1] In developed nations, the annual incidence of SCI cases ranges from 11.5 to 53.4/million, while in North America alone, over 1 million population is affected, with a lifetime cost of approximately $1.1–4.6 million USD each.[2] The annual incidence of SCI is 15–40 cases/million people.[3]

For medical professionals, it is the most severe and challenging traumatic neurological condition to treat. Around the globe, the total number of people is increasing day by day with SCI because of increasing populations, around the world, the main causes of SCI are being road traffic accident and fall from a height. Hence, the main priority to decrease its incidence is the prevention of falls and improvisation in road safety protocols.[4] As per national SCI statistical center, the cervical spine is most commonly involved (50%) in SCI, with C5 being the most commonly affected vertebrae, whereas the rest of the thoracic spine (35%) and the lumbar spine (11%).[5]

Omega-3 fatty acids are long-chain polyunsaturated fatty acid whose richest source is marine and plant diet, two major classes of omega-3 fatty acid that we mainly get from marine sources are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).[6]

As nutritional interventions are easy to administer and cost-effective, most of the studies were specific nutritional supplement based, the role of combined nutritional supplementation enhancing neurological recovery must be explored.

Aims and objectives

To elucidate the role of omega 3 fatty acid supplementation in neurological recovery of Acute Spinal Cord Injury (ASCI) subjects.

Time dependent evaluation of the level of omega 3 fatty acid and its correlation with neurological status in subjects with ASCI.

MATERIALS AND METHODS

This prospective longitudinal cohort study was carried out at the Department of Orthopaedic Surgery, King George's Medical University, Lucknow for 1 year after obtaining ethical clearance (1X-PGTSC-11A/PS) and informed consent, 26 patients with SCI in the state of Uttar Pradesh and the accompanying northern part of the country were included as per the inclusion criteria.

Inclusion criteria

Age 18–65 years of either gender, acute spinal cord injury (ASCI) <3 weeks of injury, Complete motor injury (AIS-A, B), thoracolumbar injury classification and severity ≥4 requiring surgical stabilization.

Exclusion criteria

Other associated injuries such as major thoracoabdominal injuries and head injuries needing intervention. Neuropsychiatric patients and patients on steroid or other immunosuppressant therapy or other co-morbid conditions make subjects unfit for surgery. Subjects who do not give consent to participate in the study.

Group allocation

26 ASCI subjects confirming the inclusion criteria were allocated into two treatment groups (1 and 2) based on treatment strategies:

Group 1=Conventional group (Managed by conventional method (posterior instrumentation with pedicle screw-rod system).

Group 2=Intervention group (Conventional as in Group 1 and in addition, [465 mg DHA+75 mg EPA] omega-3 fatty acid supplementation orally per day for 3 months).

Follow-up and outcomes measures: Each ASCI subject was followed for 6 months. During the study, at the time of admission, besides screening, there were 2 scheduled visits, namely, at 3rd and 6 months. Clinical assessment of ASIA scores and ASIA impairment scale and quality of life (QoL) spinal cord independence measure (SCIM) was recorded at the time of admission and at all visits. Omega-3 FA levels in serum were evaluated at baseline, 3 months, and 6th months. Correlation of serum samples at different time points with neurological recovery was done.

Clinical assessment of QoL was done at all follow-up through SCIM.

Statistical analysis

Data were analyzed by SPSS version 26 (SPSS Inc., Chicago, IL, USA). The continuous variables were evaluated by mean (standard deviation) or range value when required. The dichotomous variables were presented in number/frequency and were analyzed using the Chi-square test. For comparison of the means between the two groups, analysis by Student t-test was used. P<0.05 or 0.001 was regarded as significant.

Sample size

The sample of the study includes 26 cases of TASCI. Other criteria of the cases will be as stated in the inclusion criteria. The sample size is determined using the following formula (W.W. Daniel (ed.), Biostatistics: A foundation for analysis in the health sciences, 7th ed., John Wiley and Sons, New York, 1999).

n=(Za/2) 2 P (1−P)/d^2
n=Sample size, P=Prevalence, α=Error, d=Degree of freedom
Za/2=Differentiation coefficient (1.96 or 2).
P=6%
Prevalence in ASIA=6%=0.06 (Srivastava et al., 2018; prevalence of ASCI)
1−P=1−0.6=0.94
Za/2=1.96 (Value of standard normal valuable at 5%)
d=10%
n=1.96×1.96×0.06×(−0.06)/$(0.1)^2$
=0.2256/(0.1)2=22.56~23
n=23, Including 3 cases considering for follow-up loss-n=23+3=26

Thus sample size of the study is 26 subjects (Group 1 having 13 subjects and Group 2 having 13 Subjects).

RESULTS

The majority of the patients in Group 1 were aged between 18 and 27 years (07 [53.85%]), followed by 28–37 years (4 [32.00%]). Similarly, in Group II majority of the patients were aged between 28 and 37 years (05 [38.46%]), followed by 18–27 years and 38–47 (03 [23.00%]). Statistically, a non-significant difference was observed in the age distribution of enrolled patients (P=0.3848). Male preponderance was observed among enrolled patients in both groups. In Group I male were (08 [61.54%]) and females were (05 [38.46%]). Similarly, in Group II, males were (09 [69.23%]) and females were (04 [30.77%]). Statistically, a non-significant difference was observed in the gender of enrolled patients (P=0.6802).

The dominance of single was observed among enrolled patients in both groups. In Group I single were (09 [69.23%]) and married were (04 [30.77%]). Similarly, in Group II single were (10 [76.92%]) and married were (03 [30.77%]). Statistically, a non-significant difference was observed in the marital status of enrolled patients (P=0.6584).

In Group I, maximum patient mode of injury was fall from height (09 [69.23%]) followed by Assault and RTA (02 [15.38%]). Similarly, in Group II, maximum number of patients met RTA (07 [53.85%]) followed by fall from height (06 [46.15%]). Statistically, a non-significant difference was observed in the mode of injury of enrolled patients (P=0.0680).

In Group I, maximum patients were illiterate (06 [46.15%]) followed by Graduate (03 [23.08%]). Similarly, in Group II, the maximum patients were illiterate (07 [53.85%]) followed by primary and high school (02 [15.38%]). Statistically, a non-significant difference was observed in the educational status of enrolled patients (P=0.8424) (Table 1and Figure 1).

Baseline omega-3 levels were comparable in both Groups 1 and II. However, Group II showed gradual increasing trends in subsequent follow-ups and the difference was statistically significant (P<0.0001*) (Table 2).

While applying multiple comparison tests, a non-significant mean difference of omega 3 at baseline between cases and controls was observed. However, there is a gradual increase in omega-3 levels observed in the interventional group (Group II) was observed. Furthermore, the mean differences of omega 3 were significantly increases

Table 1: Demographic data of all enrolled subjects

S. no.	Group-1		Group-2		P-value
	n	%	n	%	
Age distribution (years)					
18–27	7	53.85	3	23.08	X=3.044
28–37	4	30.77	5	38.46	P=0.3848
38–47	1	7.69	3	23.08	
48–57	1	7.69	2	15.38	
Grand total	13	100.00	13	100.00	
Gender					
Female	5	38.46	4	30.77	X=0.1699
Male	8	61.54	9	69.23	P=0.6802
Grand total	13	100.00	13	100.00	
Marital status					
Married	4	30.77	3	23.08	X=0.1955
Single	9	69.23	10	76.92	P=0.6584
Grand total	13	100.00	13	100.00	
Mode of injury					
Assault	2	15.38	0	0.00	X=5.378
Fall from height	9	69.23	6	46.15	P=0.0680
RTA	2	15.38	7	53.85	
Grand total	13	100.00	13	100.00	
Educational status					
Illiterate	6	46.15	7	53.85	X=1.410
Primary	1	7.69	2	15.38	P=0.8424
High school	2	15.38	2	15.38	
Intermediate	1	7.69	1	7.69	
Graduate	3	23.08	1	7.69	
Grand total	13	100.00	13	100.00	

Table 2: OMEGA-3 intake status of enrolled patients

Omega3 index (%)	Group-I		Group-II		P-value
	Mean	SD	Mean	SD	
Baseline	3.11	0.71	3.27	0.65	P>0.05
At 3 months	-	-	4.98	0.84	-
At 6 months	-	-	6.75	0.75	-
			P<0.0001*		

Significance is (p<0.0001*)

Table 3: Multiple comparison test of serum OMEGA 3 level in cases (at different follow ups) and controls

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	P-value
Group-I versus Group-II (baseline)	−0.1600	−0.9332–0.6132	0.9459
Group-I versus Group-II (at 3 months)	−1.870	−2.643–−1.097	<0.0001*
Group-I versus Group-II (at 6 months)	−3.640	−4.413–−2.867	<0.0001*
Group-I (baseline) versus Group-I (at 3 months)	−1.710	−2.483–−0.9368	<0.0001*
Group-I (baseline) versus Group-I (at 6 months)	−3.480	−4.253–−2.707	<0.0001*
Group-I (at 3 months) versus Group-I (at 6 months)	−1.770	−2.543–−0.9968	<0.0001*

Significance is (p<0.0001*)

Table 4: ROC analysis of serum OMEGA 3 level between cases (at 3 months) and controls

Area under the ROC curve	Values
Area	0.9586
Std. Error	0.03507
95% confidenceinterval	0.8898–1.000
P-value	<0.0001*
Cut-off	>3.900
Sensitivity	92.31%
Specificity	84.62%

ROC: Receiver operating characteristic, Significance is (p<0.0001*)

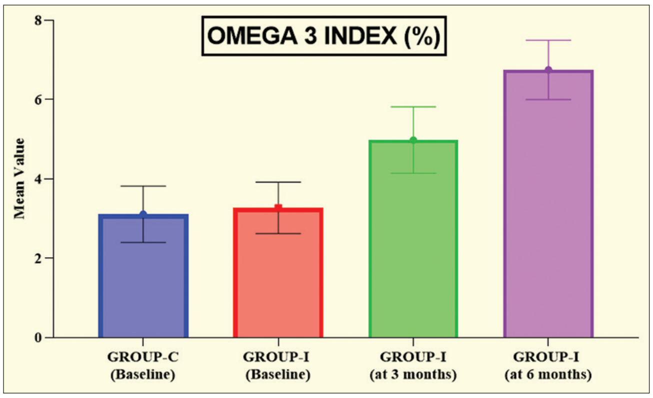

Figure 1: Omega-3 intake status of enrolled patients

with respect to controls and previous follow-up cases (P<0.0001*) Table 3.

While receiver operating characteristic analysis of omega 3 level between cases (at different follow-ups) and controls, a significant cutoff value of 3.90 with sensitivity of 92.31% and specificity of 84.62% were observed. This observation implies that at 3rd month of post-trauma, the omega-3 level above >3.90 showed a good prognosis of the patients. However, below that cutoff patients should provide extra supplementations of Omega 3 (Table 4 and Figure 2).

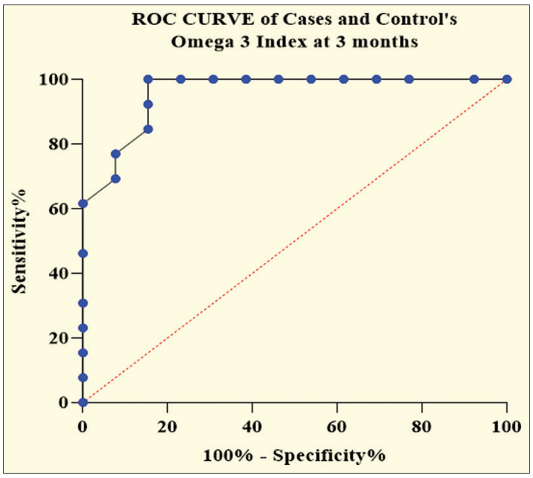

Figure 2: Receiver operating characteristic analysis of serum omega 3 level between cases (at 3 months) and controls

In both the groups increasing trend was observed for self-care management. In Group I, at baseline mean subscale was 0.00±0.00, followed by at 3 months 2.15±0.77 and at 6 months 3.85±0.66. Similarly, in Group II, at baseline mean subscale was 0.00±0.00, followed by at 3 months 4.00±0.00 and at 6 months 6.33±0.94. Statistically, a significant difference was observed at 3 and 6 months (Table 5).

In both the group's increasing trend was observed respiration and sphincter management subscale. In Group I at baseline mean subscale was 10.00±0.00, followed by at 3 months 10.69±1.59 and at 6 months 15.92±1.49. Similarly, in Group II at baseline mean subscale was 10.00±0.00, followed by at 3 months 20.42±5.18 and at 6 months 21.50±1.38. Statistically, a significant difference was observed at 3 and 6 months (Table 5).

In both the groups increasing trend of mobility subscale was observed. In Group I at baseline mean subscale was 10.00±0.00, followed by at 3 months 6.62±1.44 and at 6 months 7.92±1.14. Similarly, in Group II at baseline mean subscale was 10.00±0.00, followed by at 3 months 9.17±0.99 and at 6 months 11.08±1.32. Statistically, a significant difference was observed at 3 and 6 months Table 5.

A positive correlation was observed in both the cases while analysing the correlation among omega-3 index (at 6 months) versus the self-care subscale and Respiration-sphincter management subscale. In the case of omega 3 index (at 6 months) versus Self-care subscale (at 6 months), a positive

Table 5: Analysis of self-care subscale, respiration, and sphincter management subscale, and mobility subscale at baseline and follow-up

Time	Group-I		Group-II		P-value
	Mean	SD	Mean	SD	
Self-care subscale					
Baseline	0.00	0.00	0.00	0.00	-
At 3 months	2.15	0.77	4.00	0.00	t=8.663, P<0.0001*
At 6 months	3.85	0.66	6.33	0.94	t=7.785, P<0.0001*
Respiration and sphincter management subscale					
Baseline	10.00	0.00	10.00	0.00	-
At 3 months	10.69	1.59	20.42	5.18	t=6.474, P<0.0001*
At 6 months	15.92	1.49	21.50	1.38	t=9.906, P<0.0001*
Mobility subscale					
Baseline	0.00	0.00	0.00	0.00	-
At 3 months	6.62	1.44	9.17	0.99	t=5.261, P<0.0001*
At 6 months	7.92	1.14	11.08	1.32	t=6.532, P<0.0001*

SD: Standard deviation, Significance is (p<0.0001*)

Table 6: Correlational analysis Omega 3 index (at 6 months) versus Self-care subscale (at 6 months) and Omega3 index (at 6 months) versus Respiration and sphincter management subscale (at 6 months)

Correlation analysis	Omega 3 index (at 6 months) versus Self-care subscale (at 6 months)	Omega-3 index (at 6 months) versus Respiration and sphincter management subscale (at 6 months)
Spearman r	0.7632	0.8191
95% confidence interval	0.5179–0.8925	0.6194–0.9193
P-value	<0.0001*	<0.0001*

Significance is (p<0.0001*)

correlation (r=0.7632) was observed with a significant P<0.0001*. Similarly in the case of the omega 3 index (at 6 months) versus respiration and sphincter management subscale (at 6 months), positive correlation (r=0.8191) was observed with significant P<0.0001* (Table 6 and Figure 3).

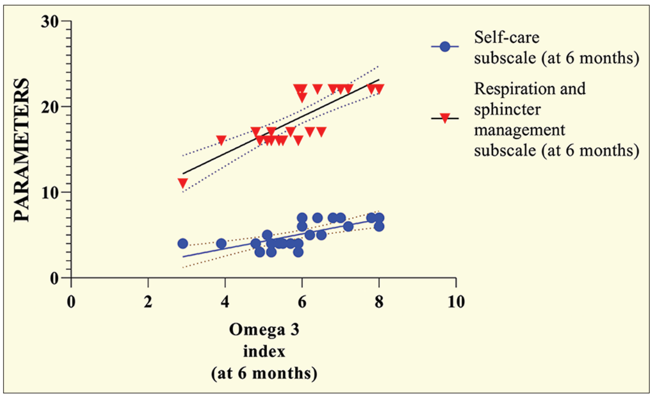

Figure 3: Graphical presentation for correlational analysis omega 3 index (at 6 months) versus self-care subscale (at 6 months) and omega 3 index (at 6 months) versus respiration and sphincter management subscale (at 6 months)

DISCUSSION

SCI is one of the most severe injuries, and it is the most challenging condition for specialists to handle and lowers the victim's QoL. Previous studies have been discussed the neuroprotective effect of omega-3 fatty acids in multiple sclerosis (MS), and Neuroprotective effect of omega-3 PUFAs has been discussed in the induction of neuro-recovery,[6] In patients with meibomian gland dysfunction, MS and hemodialysis patients,[7-9] The neuroprotective and anti-inflammatory properties of PUFA has helped in improving their QoL. However, in humans, the neuroprotective effect of omega fatty acid has not been described yet, only one of the study by Sabour et al.,[10] in their randomized controlled trial had investigated that there is an insignificant role of giving omega 3 FA for 14 months in all parameters of HR-QoL assessed by short-form (SF-36) questionnaire it was due to administration of omega 3 fatty acid in the stable phase of SCI. In the acute phase of SCI, the inflammatory reactions are at their peak level so the administration of PUFA in the acute phase of SCI is more effective due to its anti-inflammatory and neuroprotective effect. In our present study, only patient in the acute phase of injury has been enrolled having injury duration <3 weeks, In the present study, SCIM scores raised through time in Group II in comparison to Group I. More serious loss-of-function occurs in subjects with complete Paraplegia may contribute to lower QoL among these patients, The results are conflicting with completeness of injury on QoL. Few studies have shown to have better QoL in incomplete injury patients. However, in our study, we investigated improvement in QoL status in subjects with complete Paraplegia during 6-month follow-up in the Interventional group in comparison to the control group.

The increased locomotor function, reducing inflammation, and faster regeneration of damaged nerve tissue even after complete injury confirms ω-3 fatty acids therapeutic role in animal models, The ω-3 fatty acids have not achieved conclusive evidence of their therapeutic role in SCI in humans despite the presence of convincing literature data and an in-depth understanding of biological processes at the cellular and tissue levels (Table 1). A noticeable lack of standardization in the dosage of omega-3 fatty acids administered to subjects across the mentioned studies; whereas positive effects of omega 3 fatty acid have been reported in experimental SCI model some of them have been mentioned above, Although it needs more clinical trials to prove its efficacy as human trials are still lacking on its supplementation effect in SCI cases, except only few studies by Javierre et al., and Sabour et al., although they also won't get any Significant improvement in AIS scores, The key reason for this finding is worth noting. However, in the present study, significant improvement was found, likely due to the enrolled subjects having durations of injury of no more than 3 weeks. This shorter interval may have enabled greater responsiveness to treatment.

In the present study, the majority of the patients in Group I were aged between 18 and 27 years (07 [53.85%]), followed by 28–37 years (4 [32.00%]). Similarly, in Group II majority of the patients were aged between 28 and 37 years (05 [38.46%]), followed by 18–27 years and 38–47 (03 [23.00%]). Statistically, a non-significant difference was observed in the age distribution of enrolled patients (P=0.3848). The findings show that young people are more vulnerable to SCI. They may lead more active, brave, and aggressive lifestyles.[5]

In the present study, male preponderance was observed among enrolled patients in both groups. In Group I male were (08 [61.54%]) and females were (05 [38.46%]). Similarly, in Group II, males were (09 [69.23%]) and females were (04 [30.77%]). Statistically, a non-significant difference was observed in the gender of enrolled patients (P=0.6802). This may be a result of a social structure that is dominated by men the same result was investigated by Singh et al.,[11] and the same findings were reported by DeVivo et al.[12]

In the present study, single dominance was observed among enrolled patients in both groups. In group-I single were (09 [69.23%]) and married were (04 [30.77%]). Similarly, in Group II, single were (10 [76.92%]) and married were (03 [30.77%]). Statistically, a non-significant difference

Table 7: Human studies that have assessed the effects of omega-3 fatty acids in the treatment of spinal cord injury

References and study	Number of cases (intervention group/ control group) Sex, women♀/ men♂intervention group/ control)	Diagnosis	Diet and intervention	Reported results (intervention group vs. control group) (ratio) Mean age±SD, a year's group)
Present study, 2022, prospective cohort study	n=26 (13 Patients managed with the conventional method and 13 patients by conventional+(465 mg DHA+75 mg EPA)	SCI patients	465 mg DHA+75 mg EPA per day	Significant improvement in AIS scores in conventional+omega treating patients.
Norouzi Javidan et al.,[13] Double-blinded randomized clinical trial	n=104 (54/50) 19/85 (0.22) 51.15±13.43/54.12±11.76	SCI and post-injury duration longer than one year	Two capsules (435 mg of DHA and 65 mg of EPA) daily for 14 months	No significant results on UK FIM+FAM scores
Javierre et al.,[14] Prospective study	n=21 (21/0) SCI with 8.5 years mean 0/21 (0) 34.00 (average)	SCI with 8.5 years mean time since the accident	1.5 g DHA, 0.6 g EPA and 9 mg alpha-tocopherol daily for 6 months	Significant increase in training time for both upper limbs, increased gait time and increased tolerance to exercise Load.
Sabour et al.,[10] double-blinded randomized clinical trial	n=104 54 Patient having mean age 51.15±13.43 years, 50 patients having mean age 54.12±11.76 year	SCI and post-injury duration longer than 1 year	Two capsules (435 mg of DHA and 65 mg EPA) daily for 14 months	No Significant improvement in any domain of SF 36 questionaire (short health survey)
Javierre et al.,[15] prospective study	n=19 (19/0)	SCI and post-injury duration longer than 1 year	1.5 g of DHA and 0.75 g of EPA daily for 6 months	No signifcant results indicating infuence on plasma lipid profile.
Sabour et al.,[16] A double blind randomized clinical trial	n=75 (39/36) 13/69 (0.19) 40.11±14.55/38.36±12.28	SCI and post-injury duration longer than 1 year	Two capsules (435 mg of DHA and 65 mg of EPA) daily for 4 months	No significant effect on inflammatory markers, bone resorption stimulating factors and BMD
Sabour et al.,[17] Double-blinded randomized clinical trial	n=104 (54/0) 19/85 (0.22) 51.15±13.43/54.12±11.76	SCI and post-injury duration longer than 1 year	Two capsules (465 mg of DHA and 63 mg EPA) daily for 14 months	No significant results indicated a positive effect on the concentrations of leptin and adiponectin

SCI: Spinal cord injury, EPA: Eicosapentaenoic acid, DHA: Docosahexaenoic acid, SD: Standard deviation, Significance is (p<o.0001*)

was observed in the marital status of enrolled patients (P=0.6584). Similarly, according to Turczyn et al., 2022, a total of 82 patients (n=82), single dominance was observed among enrolled patients.

In the present study, in Group I, the maximum number of patients was illiterate (06 [46.15%]), followed by graduates (03 [23.08%]). Similarly, in a Group II, maximum patients were illiterate (07 [53.85%]) followed by primary and high-school (02 [15.38%]). Statistically, a non-significant difference was observed in the educational status of enrolled patients (P=0.8424).

In the present study, in both, the group's increasing trend of self-care subscale was observed. However, Group II showed comparatively higher scores as compared to Group I (P<0.0001). However, according to Sabour et al.,[10] during 14 months of administration of omega there was no beneficial effect of omega 3 fatty acid supplementation has been found in any domain of SF-36 healthy survey (SF-36) during the whole trial.

Our study's primary goal is to study the neuroprotective effect of omega-3 fatty acid supplementation in acute SCI subjects. Based on the findings of the present study, we may say that omega-3 fatty acid supplementation helps in improving the QoL of SCI Subjects by enhancing their SCIM score. The potential sedative effects and their interaction with other medicines are still being studied.

Further, current research results might provide a vital novel field for researchers to study functional and clinical outcomes of omega-3 fatty acid supplementation in acute SCI subjects. However, to bypass the confounder, we may recommend well-designed, resilient, multicentric study with a large descriptive size Table 7.

Limitations of the study

Our study was conducted on small sample size and on single tertiary care centre thus in future Larger sample size and multicentric analysis with high precision and accuracy may be recommended for a more reliable interpretation of results.

CONCLUSION

Based on the findings of this study, we can extrapolate that ω-3 fatty acids might ultimately prove to be an effective element in treating and preventing SCI. Significant correlations were found between omega three indexes versus self-care, respiration, and sphincter management and mobility subscale.

However, further to enhance the accuracy of the present findings and bypass the confounders, we recommend a resilient, multicentric study with high large sample size. In addition, periodic surveys should be done for any change or update in the pattern of results.

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KAA- Definition of intellectual content, literature survey, prepared first draft of manuscript, implementation of study protocol, data collection, data analysis, manuscript preparation and submission of article; **PP**- Concept, design, clinical protocol, manuscript preparation, editing, and manuscript revision; **ZA**- Manuscript writing, design of study, statistical analysis and interpretation; **MSR**- Review manuscript; **SV**- Literature survey and preparation of figures, tables and graphs; **SW**- Review manuscript; **MY**- Coordination and manuscript revision.

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