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Research Article

Assessing Heterosis for Grain Yield and Related Traits in Six-Row Barley (*Hordeum vulgare* L.) under Varying Sowing Conditions

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Abstract:

The present study was conducted to assess the magnitude of heterosis for grain yield and its component traits in six row barley (*Hordeum vulgare* L.) using a modified Triple Test Cross analysis with fifteen lines and three testers (RD 2508, RD 3005, and their F_1 hybrid RD 2508 × RD 3005). A total of 63 barley genotypes, including 15 lines, 3 testers, and 45 F_1 crosses, were evaluated during the 2022–23 *Rabi* season under three sowing environments (early, normal, and late) using a randomized block design with three replications. Pooled ANOVA showed significant genotypic differences across all traits studied. Across all environments, the crosses BG 105 × RD 2508, DWRUB 64 × RD 2508, PL 751 × RD 2508, RD 2035 × RD 2508, RD 2552 × RD 2508, BH 959 × RD 3005, HUB 113 × RD 3005, JB 110 × RD 3005, RD 2552 × RD 3005, RD 2907 × RD 3005, BG 105 × F_1, DWRUB 64 × F_1, DWRB 137 × F_1, RD 2035 × F_1, RD 2552 × F_1, and RD 2660 × F_1 exhibited significant heterobeltiosis for grain yield per plant in all three environments. These crosses can therefore be considered promising for enhancing barley yield under early, normal, and late sowing conditions.

Keyword: Barley, Heterosis, Heterobeltiosis, Grain yield

1. Introduction

Barley (*Hordeum vulgare* L.) was first domesticated approximately 10,000 years ago from its wild progenitor, *Hordeum vulgare* ssp. *spontaneum*, in the Fertile Crescent of the Middle East (Potla *et al.*, 2013). It belongs to genus *Hordeum,* within the Triticeae tribe of the Poaceae family. The genus Hordeum includes approximately 32 species, all characterized by a basic chromosome number of x = 7, whereas the Poaceae family as a whole comprises around 350 species. After maize, rice, and wheat, barley is the fourth most significant food crop in the world (FAO, 2020; Shabrangy *et al.*, 2021). Barley is a winter cereal crop that reproduces sexually through self-pollination and cleistogamy, and is extensively cultivated in globally. Barley serves primarily as a source of animal feed, a key ingredient in malting for beer production, and a staple food grain in many regions worldwide. Barley grains consist primarily of carbohydrates (78%), with notable proportions of protein (10%), crude fiber (5%), -glucan (3.9%), calcium (2%), and a small amount of fat (1%) (Shaveta *et al.*, 2019). Due to its low input requirements, capacity to adapt to a variety of environmental situations, and resistance to abiotic stresses such drought, salt, alkalinity, and poor soil quality, barley is highly valuable. These attributes contribute to its widespread cultivation, making it one of the most extensively grown cereal crops across both temperate and tropical regions worldwide (Yadav *et al.*, 2021).

The examination of heterosis assists plant breeders in early generation selection, enabling the elimination of less productive crosses (Lal *et al.*, 2018a). The magnitude of heterosis gives information on extent of genetic diversity in the parents of cross and aids in selecting superior parents for crosses to exploit hybrid vigor. Heterosis refers to allelic or non-allelic interaction of genes influenced by specific environments. Barley is self-pollinated crop and there is currently no suitable method for producing hybrid seeds on a commercial scale. Therefore, the heterosis *per se* may not be of economic value in this crop at present. However, understanding the degree and magnitude of heterosis is essential for choosing the most ideal crosses to produce good segregants in future generations and for determining the outcome of future breeding programs to further improve barley grain yield.

2. Materials and Methods

The experimental materials of the current investigation comprised 17 genetically diverse genotypes and one F_1 hybrid of 6-row feed barley (*Hordeum vulgare* L.), procured from the All India Coordinated Wheat and Barley Improvement Project (AICW&BIP) at the RARI, Durgapura. The experiment was carried out at Sri Karan Narendra College of Agriculture's Instructional Farm in Jobner, Rajasthan, during the *Rabi* seasons of 2021–22 and 2022–23. Crosses were carried out using the modified Triple Test Cross (TTC) design proposed by Ketata *et al.* (1976), employing two genetically diverse 6-row feed barley genotypes, RD 2508 and RD 3005, along with their F_1 hybrid (RD 2508 × RD 3005), which were designated as testers T_1, T_2, and T_3, respectively. During the *Rabi* season of 2021–22, a crossing block was established where the three testers (T_1, T_2, and T_3) served as male parents and were crossed with 15 genetically diverse 6-row feed barley genotypes (designated L_1 to L_{15}), which acted as female parents, resulting in the development of 45 crosses, including 30 single crosses and 15 three-way crosses. The current experimental material, consisting of 63 genotypes including three testers, fifteen lines, and forty-five F_1 crosses was assessed during the *Rabi* season of 2022–2023 using a randomized block design (RBD) with three replications under three different sowing environments: early (E_1), normal (E_2), and late (E_3). Each genotype was allotted two rows, each 2.5 meters in length with row spacing of 30 cm, while a uniform plant-to-plant distance of 10 cm was maintained across all environments. Observations were recorded for fourteen traits, namely: days to 75% heading, days to maturity, plant height (cm), effective tillers per plant, grains per spike, spike length (cm), 1000-grain weight (g), grain yield per plant (g), biological yield per plant (g), harvest index (%), protein content (%), starch content (%), proline content (µg/100 mg fresh weight), and chlorophyll content (SPAD). Data for all fourteen traits were collected from ten randomly selected plants in individual plot of the F_1 progenies and parental lines across all replications and environments, except for days to 75% heading, days to maturity, and 1000-grain weight, which were recorded on a plot basis.

Analysis of variance (ANOVA) for all traits in each environment was performed according to the methodology described by Panse and Sukhatme (1985). Heterosis (H%) and heterobeltiosis (HB%) were calculated as the deviation of the F_1 hybrid value from the mid-parent and better-parent values, following the methods described by Shull (1914) and Fonseca and Patterson (1968), respectively.

3. Results and Discussion

Significant differences were found among genotypes for all traits, both individually and across environments, according to the experimental design's pooled analysis of variance (Table 1). With the exception of harvest index and starch content, the mean square resulting from the genotype × environment interaction was significant for every trait. For all traits except 1000-grain weight, harvest index, protein content, and starch content, the mean sum of squares resulting from the parent × environment interaction was significant. The mean sum of squares due to cross × environment interaction was significant for all traits except harvest index and starch content. This indicates the existence of sufficient genetic variability and heterosis in the experimental material used in the present investigation. Similar results were reported by Singh *et al.* (2007), Lodhi *et al.* (2015), Patial *et al.* (2016), and Lal *et al.* (2018b). Although the magnitude and direction of heterosis differ across crosses and traits, this research paper focuses exclusively on the trait grain yield per plant.

Table 1: Pooled ANOVA showing mean squares of parents and F_1's for different traits

Source	Env.	Gen.	Parent	P v/s C	Cross	G x E	P x E	Cross x E	Pooled Error
df	2	62	17	1	44	124	34	88	372
Days to 75% heading	10325.19**	87.69**	48.52**	1614.93**	68.11**	10.86**	12.22**	10.19**	2.48
Days to maturity	6104.13**	69.33**	24.99**	1810.51**	46.89**	9.53**	10.78**	8.53**	3.77
Plant height (cm)	15520.61**	161.12**	217.98**	846.95**	123.56**	11.52**	11.55*	11.50**	7.67
Effective tillers per plant	245.94**	26.61**	32.26**	162.11**	21.35**	0.81**	1.26**	0.63**	0.18
Grains per spike	11603.02**	258.82**	211.07**	6167.46**	142.98**	15.76**	13.73**	16.22**	6.65
Spike length (cm)	99.16**	9.77**	8.24**	125.05**	7.74**	0.34**	0.40**	0.24**	0.13
1000-grain weight (g)	3390.20**	91.08**	36.58**	2284.94**	62.28**	2.96**	2.13	2.56**	1.59
Grain yield per plant (g)	1636.57**	70.20**	51.47**	992.17**	56.49**	2.27**	2.60**	1.82**	0.92
Biological yield per plant(g)	4150.93**	176.23**	82.13**	3228.96**	143.20**	8.14**	7.03**	7.61**	2.78
Harvest index (%)	1995.33**	157.14**	237.08**	670.42**	114.59**	9.83	9.15	10.14	9.62
Protein content (%)	22.53**	1.98**	2.22**	3.79**	1.84**	0.07**	0.04	0.08**	0.05
Starch content (%)	166.29**	7.65**	5.67**	14.27**	8.27**	0.4	0.07	0.54	0.43
Proline content (µg/100mg)	6491.10**	535.96**	614.31**	31.96**	517.14**	3.56**	3.20**	2.64**	1.27
Chlorophyll content (SPAD)	2892.80**	44.59**	54.55**	217.61**	36.80**	4.98**	4.15**	5.29**	1.53

*, ** Significant at 5% and 1%, respectively

In the current study, two types of heterosis were investigated: average heterosis and heterobeltiosis. Heterosis as percent increase (+) or decrease (-) of F_1 over mid parent (heterosis) and better parent (heterobeltiosis) were computed for all the fourteen traits in all three environments. Enhancing grain yield remains the primary objective of breeding programs, as it is closely linked to favorable heterotic effects. Consequently, significant positive heterosis and heterobeltiosis are highly desirable. In the current research study, mid-parent heterosis for grain yield per plant ranged from -4.04% (JB 110 × RD 2508) to 41.79% (RD 2660 × F_1) in environment E_1; from -3.46% (DWRB 137 × RD 2508) to 44.79% (RD 2660 × F_1) in E_2; and from -10.87% (RD 2052 × F_1) to 49.47% (DWRUB 64 × F_1) in E_3. Out of 45 crosses evaluated in each environment, significant positive heterosis over the mid-parent was observed in 40 crosses in E_1, 44 crosses in E_2, and 34 crosses in E_3. Significant positive heterobeltiosis for grain yield per plant ranged from 7.08% (DWRB 137 × RD 3005) to 40.81% (DWRUB 64 × RD 2508) in environment E_1; from 6.19% (PL 751 × F_1) to 39.23% (DWRUB 64 × RD 2508) in E_2; and from 8.86% (RD 2660 × RD 3005) to 38.16% (DWRUB 64 × RD 2508) in E_3. Out of 45 crosses evaluated in each environment, significant positive heterobeltiosis was observed in 20 crosses in E_1, 27 in E_2, and 19 in E_3 (Table 2). El-Aty *et al.* (2011), Vishwakarma *et al.* (2011), Saad *et al.* (2013), Pesarakhlu *et al.* (2016), Ram and Shekhawat (2017b), Parashar (2019a), Lal *et al.* (2020), Meena *et al.* (2022), Panwar *et al.* (2022) and Sharma *et al.* (2022) also reported similar findings in different environments for grain yield per plant.

The superiority of hybrids over the better-performing parent (heterobeltiosis) is a key criterion for evaluating the commercial potential of heterosis. It also helps in identifying parental combinations that are likely to produce highly transgressive segregants. The examination of Table 2 revealed that the crosses BG 105 x RD 2508, DWRUB 64 x RD 2508, PL 751 x RD 2508, RD 2035 x RD 2508, RD 2552 x RD 2508, BH 959 x RD 3005, HUB 113 x RD 3005, JB 110 x RD 3005, RD 2552 x RD

Table 2: Heterosis (H) and heterobeltiosis (HB) estimates for grain yield per plant across individual environments

Crosses	Heterosis (H)			Heterobeltiosis (HB)		
	E_1	E_2	E_3	E_1	E_2	E_3
BG 105 × RD 2508	27.81**	35.51**	26.18**	23.53**	31.98**	25.79**
BH 959 × RD 2508	6.71*	16.22**	-2.98	4.87	15.12**	-
DWRUB 64 ×RD 2508	41.09**	42.37**	40.97**	40.81**	39.23**	38.16**
DWRB 137 × RD 2508	-0.11	-3.46	-4.74	-	-	-
HUB 113 × RD 2508	5.64	7.89**	-2.42	-	-	-
JB 110 × RD 2508	-4.04	6.35*	20.09**	-	-	-
K 551 × RD 2508	13.76**	18.30**	12.04**	-	2.12	-
NDB 1445 × RD 2508	13.12**	11.75**	2.30	-	-	-
PL 751 × RD 2508	21.12**	22.51**	32.97**	15.44**	17.52**	23.02**
VLB 118 × RD 2508	16.85**	14.78**	15.91**	-	-	-
RD 2035 × RD 2508	39.93**	40.23**	48.82**	20.49**	18.88**	30.57**
RD 2052 × RD 2508	32.47**	38.08**	44.10**	3.85	12.14**	18.99**
RD 2552 × RD 2508	33.20**	35.91**	46.52**	30.63**	35.65**	28.55**
RD 2660 × RD 2508	20.91**	21.38**	19.15**	-	1.73	-
RD 2907 × RD 2508	14.29**	13.82**	13.66**	-	0.00	-
BG 105 × RD 3005	13.75**	9.74**	16.22**	6.09	3.05	-
BH 959 × RD 3005	25.40**	27.37**	27.39**	15.13**	15.69**	12.04**
DWRUB 64 × RD 3005	20.72**	19.38**	17.08**	8.89**	7.18**	-
DWRB 137 × RD 3005	12.20**	15.12**	9.32**	7.08*	7.89**	-
HUB 113 × RD 3005	11.76**	11.57**	20.47**	11.06**	11.57**	14.02**
JB 110 × RD 3005	17.74**	14.73**	13.58**	17.59**	11.81**	9.59*
K 551 × RD 3005	9.09**	13.47**	16.12**	5.53	6.25	3.69
NDB 1445 × RD 3005	15.57**	14.86**	5.70	3.52	-	-
PL 751 × RD 3005	13.80**	10.93**	10.34**	-	-	-
VLB 118 × RD 3005	14.79**	16.54**	4.95	5.28	6.02	-
RD 2035 × RD 3005	14.59**	20.55**	6.13	8.54*	10.65**	1.00
RD 2052 × RD 3005	25.66**	28.97**	-2.64	7.04	12.85**	-
RD 2552 × RD 3005	21.10**	20.13**	19.79**	11.39**	10.25**	14.00**
RD 2660 × RD 3005	19.02**	16.73**	16.14**	3.77	5.79	8.86*
RD 2907 × RD 3005	30.65**	30.42**	30.74**	21.13**	24.54**	23.99**
BG 105 × F_1	32.96**	34.97**	38.70**	28.48**	28.66**	20.00**
BH 959 × F_1	10.50**	15.90**	32.61**	5.04	6.81*	19.89**
DWRUB 64 × F_1	29.00**	32.86**	49.47**	20.40**	20.99**	26.81**
DWRB 137 × F_1	16.03**	20.00**	24.26**	14.84**	14.17**	10.19**
HUB 113 × F_1	15.09**	14.35**	14.23**	10.26**	12.56**	5.03
JB 110 × F_1	2.18	10.05**	-8.97*	-	5.61	-
K 551 × F_1	4.62	12.27**	-2.69	-	3.59	-
NDB 1445 × F_1	15.59**	16.69**	10.58*	0.23	-	-
PL 751 × F_1	14.08**	18.69**	13.97**	2.02	6.19*	5.62
VLB 118 × F_1	11.43**	13.25**	-0.48	-	1.57	-
RD 2035 × F_1	33.50**	38.04**	33.56**	22.14**	24.89**	31.00**
RD 2052 × F_1	26.38**	24.94**	-10.87**	4.43	7.85*	-
RD 2552 × F_1	23.37**	23.57**	35.26**	17.51**	15.09**	32.67**
RD 2660 × F_1	41.79**	44.79**	37.01**	19.81**	29.37**	24.78**
RD 2907 × F_1	15.21**	20.86**	9.50*	3.26	13.68**	0.87

*, ** Significant at 5% and 1%, respectively

3005, RD 2907 x RD 3005, BG 105 x F_1, DWRUB 64 x F_1, DWRB 137 x F_1, RD 2035 x F_1, RD 2552 x F_1 and RD 2660 x F_1 depicted significant heterobeltiosis for grain yield per plant across all three environments. As a result, these crosses could be seen as promising for increasing barley production in conditions of early, normal, and late (heat stress) sowing. The cross combinations exhibit significant desirable heterobeltiosis for grain yield per plant and its contributing traits in different environments are presented in Table 3. The parents displaying desirable heterobeltiosis for grain yield per plant also displayed desirable heterobeltiosis for at least one or more yield qualities, as was clear from studying the table. For example, heterobeltiosis for grain yield per plant was primarily influenced by days to 75% heading, plant height, days to maturity, number of effective tillers per plant, spike length, number of grains per spike, 1000-grain weight, and biological yield per plant across all three environments. These findings supported Grafius' (1959) statement that as yield is a product of the interactions between its many contributing features, there could not be a distinct gene system for yield in and of itself.

Table 3: The cross combinations displayed significant positive heterobeltiosis for grain yield per plant and its component traits across diverse environments

Environments	Crosses	Grain yield per plant (g)	Days to 75% heading	Days to maturity	Plant height (cm)	Effective tillers per plant	Grains per spike	Spike length (cm)	1000-grain weight (g)	Biological yield per plant (g)	Harvest index (%)	Protein content (%)	Starch content (%)	Proline content (µg/ 100 mg fresh weight	Chlorophyll content (SPAD)
E_1	DWRUB 64 x RD 2508	+	+	+	+	+	+	+	+	+	-	-	-	-	+
	RD 2552 x RD 2508	+	+	+	+	+	+	+	+	+	-	-	-	+	-
	BG 105 x F_1	+	+	+	+	+	+	+	+	+	-	-	-	-	+
	BG 105 x RD 2508	+	+	-	+	+	+	+	+	+	-	-	-	-	+
	RD 2035 x F_1	+	+	+	+	+	+	+	+	+	-	-	+	-	-
	RD 2907 x RD 3005	+	-	-	-	+	-	-	+	+	-	+	-	-	+
	RD 2035 x RD 2508	+	+	+	+	+	+	+	+	+	-	-	+	+	-
	DWRUB 64 x F_1	+	+	+	-	+	+	+	+	+	-	-	-	-	-
	RD 2660 x F_1	+	-	-	-	+	-	-	+	+	-	-	-	-	-
	JB 110 x RD 3005	+	+	+	+	+	+	-	+	+	-	-	-	-	-
	RD 2552 x F_1	+	-	-	-	-	-	-	+	+	-	-	-	+	-
	PL 751 x RD 2508	+	+	-	-	-	+	-	+	+	-	-	+	-	-
	BH 959 x RD 3005	+	+	+	+	-	+	+	+	+	-	-	-	-	+
	DWRB 137 x F_1	+	+	+	+	+	-	+	+	-	-	-	+	-	-
	RD 2552 x RD 3005	+	-	-	-	-	+	+	+	+	-	-	-	-	-
	HUB 113 x RD 3005	+	+	+	-	-	+	+	+	+	-	+	-	-	+
	HUB 113 x F_1	+	-	+	+	-	+	-	+	+	-	-	-	-	+
	DWRUB 64 x RD 3005	+	+	+	-	-	+	-	+	+	-	-	-	-	-
	RD 2035 x RD 3005	+	-	-	-	+	+	+	+	-	-	-	-	-	-
	DWRB 137 x RD 3005	+	+	+	-	-	+	+	+	-	-	-	-	-	+
E_2	DWRUB 64 x RD 2508	+	+	+	+	+	+	+	+	+	-	-	-	+	+
	RD 2552 x RD 2508	+	+	+	+	+	+	+	+	+	-	-	-	+	-
	BG 105 x RD 2508	+	+	+	+	+	+	+	+	+	-	-	-	+	+
	RD 2660 x F_1	+	-	-	-	+	-	-	+	+	-	-	-	-	-

	BG 105 x F_1	+	+	+	+	+	+	+	+	+	+	-	-	-	+
	RD 2035 x F_1	+	+	+	+	+	+	+	+	+	-	-	+	+	-
	RD 2907 x RD 3005	+	-	+	-	+	-	-	+	+	-	+	-	-	+
	DWRUB 64 x F_1	+	+	+	-	+	+	+	+	+	-	-	+	-	-
	RD 2035 x RD 2508	+	+	+	+	+	+	+	+	+	-	-	+	+	-
	PL 751 x RD 2508	+	+	-	-	-	+	+	+	+	-	-	+	-	-
	BH 959 x RD 3005	+	+	+	+	-	+	+	+	+	-	-	-	-	+
	BH 959 x RD 2508	+	+	+	-	-	+	+	+	+	-	-	-	-	-
	RD 2552 x F_1	+	-	+	-	+	-	-	+	+	-	-	-	+	-
	DWRB 137 x F_1	+	+	+	-	-	-	+	+	-	-	-	+	-	-
	RD2907 x F_1	+	-	-	+	+	-	-	-	+	-	-	-	-	+
	RD 2052 x RD 3005	+	-	+	-	-	+	+	+	+	-	-	-	+	-
	HUB 113 x F_1	+	-	+	+	-	+	-	+	+	-	-	-	-	+
	RD 2052 x RD 2508	+	+	+	+	+	+	+	+	+	-	-	+	-	+
	JB 110 x RD 3005	+	+	+	+	+	+	-	+	+	-	-	-	-	+
	RD 2035 x RD 3005	+	-	-	-	+	+	+	+	+	-	-	-	-	-
	HUB 113 x RD 3005	+	+	+	-	-	+	+	+	+	-	+	-	-	+
	RD 2552 x RD 3005	+	-	-	-	-	+	+	+	+	-	-	-	+	-
	DWRB 137 x RD 3005	+	+	+	-	-	+	+	+	-	+	-	-	-	+
	RD 2052 x F_1	+	+	+	-	+	-	-	+	+	-	-	-	-	+
	DWRUB 64 x RD 3005	+	+	+	-	-	+	-	+	+	-	-	-	-	-
	BH 959 x F_1	+	+	-	-	-	-	-	+	+	-	-	-	-	+
	PL 751 x F_1	+	+	+	-	-	+	+	-	+	-	-	+	-	-
E_3	DWRUB 64 x RD 2508	+	+	+	+	+	+	+	+	+	-	-	-	+	+
	RD 2552 x F_1	+	-	+	-	-	+	-	+	+	-	-	+	+	-
	RD 2035 x F_1	+	+	+	+	+	+	+	+	+	-		-	-	-
	RD 2035 x RD 2508	+	+	+	+	+	+	+	+	+	-	-	+	+	+
	RD 2552 x RD 2508	+	-	+	-	+	+	+	+	+	+	+	+	+	-
	DWRUB 64 x F_1	+	-	+	-	+	+	+	+	+	-	-	+	-	-
	BG 105 x RD 2508	+	+	+	+	+	+	+	+	+	-	-	-	+	+
	RD 2660 x F_1	+	-	+	+	+	-	-	+	+	-	+	+	-	-
	RD 2907 x RD 3005	+	+	+	-	+	-	-	+	+	-	+	-	-	+
	PL 751 x RD 2508	+	-	-	-	+	-	-	+	+	-		-	-	-
	BG 105 x F_1	+	+	+	+	+	+	+	+	+	+	-	-	-	-
	BH 959 x F_1	+	-	-	+	-	+	+	+	+	-	-	-	-	-
	RD 2052 x RD 2508	+	+	+	+	+	+	+	+	+		-	+	-	-
	HUB 113 x RD 3005	+	-	-	-	-	-	+	+	+	-	-	-	-	+
	RD 2552 x RD 3005	+	+	+	-	-	+	+	+	+	-	-	-	+	-
	BH 959 x RD 3005	+	+	+	+	-	+	+	+	+	-		-	-	+
	DWRB 137 x F_1	+	-	-	+	+	+	-	+	-	+	-	-	-	+
	JB 110 x RD 3005	+	-	-	+	+	-	-	-	+	-	+	-	-	-
	RD 2660 x RD 3005	+	+	+	+	+	-	-	+	+	-	+	-	-	-

+ Significant and desirable; - Non significant and undesirable

Heterobeltiosis for grain yield is likely due to heterobeltiosis in various yield-contributing traits. While crosses showed heterotic expression for grain yield per plant, they were not heterotic for all traits. Furthermore, it was noted that the various surroundings affected the expression of heterobeltiosis, resulting in a substantial interaction between environment and genetics for practically all traits. The findings of Sharma *et al.* (2002), Potla *et al.* (2013), Saad et al. (2013), Mansour (2016), Ram and Shekhawat (2017a), and Sharma et al. (2022) consistently support the observation of maximum heterobeltiosis for grain yield per plant across various environments and traits.

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Authors' contributions

AK, SSR, SSP and KJ- compiled and edited the manuscript; SC helped in the statistical analysis of the findings and in editing of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical Approval

The article doesn't contain any study involving ethical approval.

Use of Generative AI or AI assisted technologies

Authors declare that no Generative AI or AI assisted technologies have been used in preparation of this manuscript.

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