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Effect of Giardia duodenalis in protein malnourished and renourished mice

Research Article

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Abstract: The present study was designed to delineate the effect of *Giardia duodenalis* in malnourished and renourished BALB/c mice. Control and renourished mice were fed with a standard pellet diet while malnourished mice were fed with a low protein (4.3 %) diet both before and after being challenged orally with actively growing *G. duodenalis* trophozoites. It was observed that malnourished mice had a greater severity and longer duration of *Giardia* infection compared with renourished mice. These malnourished mice also had less body mass but higher cyst and trophozoite counts. Malnourished mice infected with *Giardia* had significantly decreased level of total serum proteins, albumin, globulins, hemoglobin, leukocyte, and differential leukocyte counts compared with renourished mice. From the data it is concluded that protein malnutrition profoundly affects the anthropometric and physiological parameters of the body indicating greater susceptibility and severity of the disease.

Keywords: *Giardia duodenalis* • Giardiasis • *Malnutrition* • *Renourished*

1. Introduction

Malnutrition is a serious health issue that requires urgent attention particularly in developing countries. It can be defined as a state in which a deficiency of energy, protein, vitamins, and minerals causes measurable adverse effects on body composition, functions or clinical outcomes [1,2]. Every year, malnutrition contributes to the death of about 5.6 million children below 5 years of age and about 146 million children in developing worlds are under weight, anemic and at higher risk of early death [3-6]. Protein energy malnutrition results when the body is stressed from starvation and is not receiving the protein, energy fuel and micronutrients needed to sustain the metabolic process required for health and survival [7]. Severe malnutrition during childhood affects thymic development, which results in long-term reduction of peripheral lymphocyte counts and represents a key factor in the increased susceptibility to infections [3].

Parasitic infections in children (below 5 years of age) are problematic because of negative lifelong health consequences and can contribute to malnutrition resulting in growth retardation [8]. *Giardia duodenalis* is a common cause of endemic and epidemic water borne diarrhea throughout the world affecting almost every individual [9]. The interactions of the parasite with the surface mucosa of small intestine have been proposed to be responsible for pathogenesis leading to malabsorption, maldigestion, and diarrhea [10,11]. However, scarce information is available pertaining to the consequences of *Giardia* infection in malnourished and renourished children. Thus, it is pertinent to investigate the effect of *G duodenalis* in malnourished and renourished animals.

2. Materials and Methods:

Parasite: *Giardia duodenalis* trophozoites (Portland Strain I) were auxenically cultured in TYI-S-33 medium supplemented with antibiotic solution and pH was adjusted to 6.9 before sterilization with 0.22 µm filter.

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Actively growing trophozoites (48-72 hrs old cultures) were harvested by chilling in ice for 15 minutes, followed by centrifugation at 2000 rpm for 15 minutes and finally resuspended in PBS (pH 7.2) to contain 5×10^6 trophozoites for inoculation of mice [12].

Animal: BALB/c mice aged 5-6 weeks old (18-20 g) were procured from the Central Animal House, Panjab University, Chandigarh, India. The mice were housed under the standard condition of light and dark cycle and were fed with the standard pellet diet, (Hindustan Lever Products, Limited, Kolkata, India) and 4.3% protein diet (Ashirwad Private Limited, Kharar Punjab, India formulated as per Villena et al. and water *ad libitum* [13]**.** The standard pellet diet comprises of proteins (230 g/kg), carbohydrates (538 g/kg), lipids (50 g/kg), vitamin mixture (22 g/kg) and mineral mixture (40 g/kg), while protein free diet contain 43 g/kg of protein and 758 g/kg of carbohydrates with the rest of the nutrients in the same composition. Before being administered, water and feed were monitored for bacterial/parasitic contamination by Gram's and Lugol's iodine, staining technique [14]. Stool specimens of all the animals were examined for three consecutive days. Only parasite free animals were employed. Care and use of animals was in accordance with the guidelines of the institutional ethical committee.

Induction of malnutrition: Normal mice were fed with a 4.3% protein diet for 21 days. At the end of this period, animals that had lost about 35-55% of their original weight were labeled as malnourished [15].

Renourishing after malnutrition: Malnourished mice were renourished with the standard pellet diet till end of the experiment and labeled as renourished [16].

Groups of animals: Animals were divided into six groups with each group comprised of 24 mice. **Group I (control)**: Normal mice were fed intra oesophageally with a single dose of 0.1 ml PBS (pH 7.2) daily for 30 days and were given standard pellet diet. **Group II (malnourished)**: These malnourished mice were fed orally with 0.1ml PBS (pH 7.2) daily and were given a 4.3% protein diet for up to 30 days. **Group III (renourished)**: Malnourished mice were fed orally with 0.1ml PBS (pH 7.2) daily and were provided with a standard pellet diet for up to 30 days. **Group IV (*Giardia*-infected)**: Normal mice were infected orally with a single dose of 5×10^6 *Giardia* trophozoites and were provided a standard pellet diet for up to 30 days. **Group V (malnourished–*Giardia*-infected)**: Malnourished animals were infected orally with a single dose of 5×10^6 *Giardia* trophozoites and were provided a 4.3% protein diet for up to 50 days. **Group VI (renourished-*Giardia*-infected):** These malnourished mice were infected orally with a single dose of 5×10^6 *Giardia* trophozoites and were given a standard pellet diet for up to 50 days.

Follow up of animals: After respective treatment, the body mass and cyst counts were analyzed. Animals were sacrificed by retro-orbital plexuses route in batches of 6 on day 7, 11, and 17 post infection (PI) respectively for the estimation of trophozoite counts, blood biochemical parameters and organ mass (liver, spleen and small intestine).

Estimation of body mass: Body mass of mice was recorded on every alternate day using an ordinary balance weight scale.

Enumeration of *Giardia* cysts in the stool: Cysts were counted in stool samples of mice as per Shukla et al. [17]. Briefly, 0.1 gm of freshly passed fecal sample was mixed thoroughly with 1 ml formal saline using pestle mortar. Cysts were stained with Lugol's Iodine and counted every alternate day.

Enumeration of *Giardia* trophozoites in the small intestine: Trophozoites were counted in the intestinal perfusate of mice as per Shukla et al [17]. After sacrificing, the proximal 10 cm section of the small intestine was removed, placed in 5ml of the ice chilled isotonic saline solution, minced, kept in ice for 15-20 minutes and finally, trophozoites were counted using haemocytometer. Mice with no detectable trophozoites were considered to have cleared the infection.

Blood biochemical parameters: Blood samples were collected from mice by retro-orbital plexuses route and the serum separated. Total serum proteins, albumin and globulin were assayed as per Dock et al. [16]. However, Hemoglobin, Differential Leukocyte Count (DLC) and Total Leukocyte Count (TLC) were performed as per Tiwari et.al. [14].

Total serum protein estimation: To 3.0 ml of the working solution (48ml of 2% Na_2Co_3 in 0.1 N NaOH and 1ml each of $CuSo_4$ (1% aqueous) and sodium potassium tartarate (2% aqueous) 1.0 ml of diluted protein sample was mixed and was kept at 37°C for 10 minutes. After incubation, 0.3 ml of Folin's reagent was added, mixed vigorously, and incubated at 37°C for 30-45 minutes. The absorbance was read at 680nm and results were expressed in µg/ml.

Albumin and Globulin estimation: For albumin estimation, to 1 ml of diluted serum 10 µl

of bromocresol green solution was added, mixed and optical density was measured at 628 nm. The results were expressed as µg/ml. However, globulin concentration was calculated as follow:

Total protein concentration – concentration of albumin

Hemoglobin estimation: To 0.02 ml of freshly drawn blood in Sahlis tube 0.1 N HCl was added upto 20 divisions, mixed thoroughly and kept for 5 to 10

minutes. After this, distilled water was added drop by drop followed by mixing between each addition until the color matched the standard. Finally, the amount of the solution in graduated tube was observed and results were expressed in gram percentage

Differential Leukocyte Count (DLC) estimation: Thin blood film was prepared from tail vein of mice after cleaning with spirit. Slides were stained with Leishman stain for 8-10 minutes, washed, dried and examined microscopically.

Total Leukocyte Count (TLC) estimation: Blood was drawn in the throma pipette containing the white bead up to the mark 1 and dilute it with the turks fluid (2% acetic acid containing 2ml of methylene blue) up to mark 2. Mixed properly and leukocyte counts were performed using haemocytometer. Results were expressed as counts/ml.

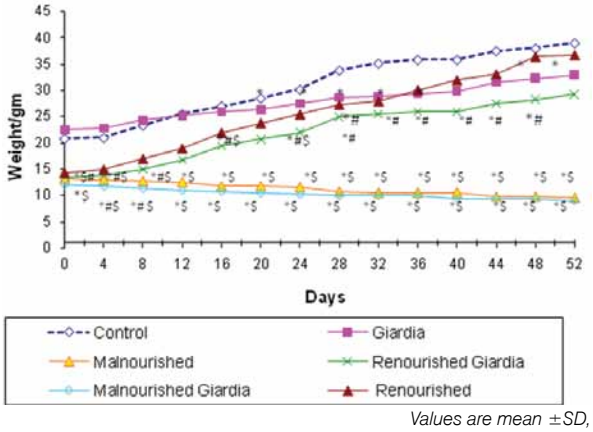
Determination of organ mass: The mice were sacrificed and their liver, spleen, and entire small intestine were removed and weighed on electronic digital balance (Sd-fine chem. Limited, SD-300, Chandigarh,India).

Statistical analysis: The inter group variation was assessed by one way analysis of variance (ANOVA) followed by Post Hoc Tests Multiple Comparison Bonferroni and statistical significance at p<0.001.

3. Results

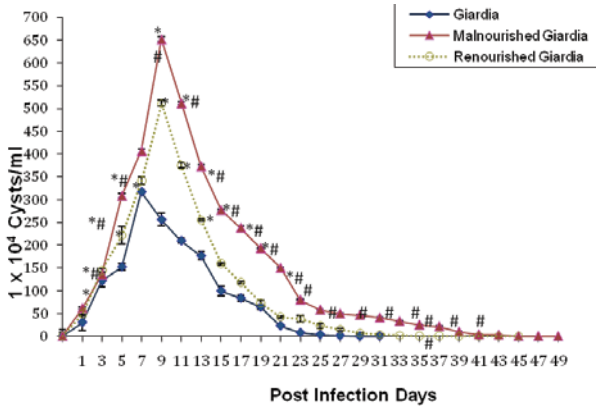
Body mass: Malnourished and renourished mice (Group II & III) had same body mass initially, but were

Figure 1. Body mass of mice belonging to different groups.



Values are mean ±SD,
**p<0.001 v/s Control,*
$p<0.001 v/s Giardia,
#p<0.001 v/s malnourished.

Figure 2. *Giardia* cysts in feces of different groups of mice.

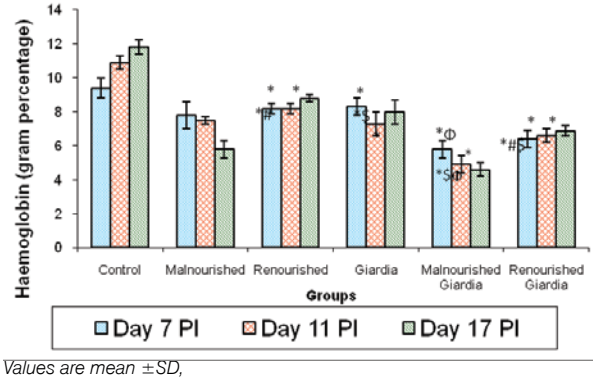


Values are mean ±SD,
**p<0.001 v/s Giardia,*
 # p<0.001 v/s renourished Giardia.

Figure 3. *Giardia* trophozoites count in small intestine of the different groups of mice on day 7, 11 and 17 Post Infection (PI).

Values are mean ±SD,
**p<0.001 v/s Giardia,*
 # p<0.001 v/s renourished Giardia.

Figure 4. Hemoglobin level in different groups of mice on different days



Values are mean ±SD,
**p<0.001 v/s Control,*
#p<0.001 v/s malnourished,
$ p<0.001 v/s renourished,
Φp<0.001 v/s Giardia.

Table 1. Total serum protein, albumin and globulin level in different groups of mice. Values are mean count±SD

Days	Day 7 PI (μg/ml)			Day 11 PI (μg/ml)			Day 17 PI (μg/ml)		
Groups	Total Serum Protein	Albumin	Globulin	Total Serum Protein	Albumin	Globulin	Total Serum Protein	Albumin	Globulin
Control	68.33±15.9	39.3±16.3	28.33±4.9	104±5.29	65.66±14.3	38.33±16.07	125±5	90±5	35±5
Malnourished	17.33±2.52*	11.13±1.8*	6.2±0.78*	11±4.58*	7.86±1.97*	3.47±3.33*	6±3.61*	3.9±2.89*	2.1±0.81*
Renourished	34±3.6*,#	8.96±3.5*,#	5.33±2.21*,#	74.33±12.8 *,#	39.33±4.51 *,#	31.67±14.46 *,#	106±12.16 *,#	83.33±12.58 *,#	22.67±6.80 *,#
Giardia	57.67±11.24 *,#,Ψ	29.13±6.45 *,#,Ψ	28.36±4.92 *,#,Ψ	79.33±5.13 *,#, Ψ	50±1 0 *,#, Ψ	29.33±15.04 *,#, Ψ	74.67±4.51 *,#, Ψ	45.67±8.14 *,#, Ψ	29±8.54 *,#, Ψ
Malnourished *Giardia*	13.27±4.62 *, Ψ, Φ	11±3.61 *, Ψ, Φ	2.3±1.1 *, Ψ, Φ	11±1 *, Ψ, Φ	4.63±2.59 *, Ψ, Φ	6.367±3.43 *, Ψ, Φ	6.67±1.53 *, Ψ, Φ	3.56±3.51 *, Ψ, Φ	1.7±1.13 *, Ψ, Φ
Renourished *Giardia*	30.33±16.62 *,#	27.33±9.1 *,#	20.33±12.88 *,#	60±10 *,#	45.33±16.1 *,#	14.66±6.42 *,#	64.33±8.15 *,#	41.33±7.09 *,#	23.3±9.7 *,#

**p<0.001 v/s Control*
#p<0.001 v/s malnourished,
Ψ p<0.001 v/s renourished,
Φ p<0.001 v/s Giardia.

significantly (p<0.001) lower compared with the control or *Giardia*-infected mice (Group I and IV, Figure 1). Interestingly, the body mass of malnourished (Group II) and malnourished- *Giardia*-infected mice (Group V) decreased significantly (p<0.001). The decrease was significant compared with their controls. However, the body mass of renourished (Group III) and renourished--*Giardia*-infected mice (Group VI) increased significantly (p<0.001) at each point of observation compared with malnourished and malnourished-*Giardia*-infected mice. However, the body mass was significantly (p<0.001) less that of control or *Giardia*-infected mice (Group I & IV, Figure 1).

***Giradia* cysts in feaces:** *Giardia*- infected mice (Group IV) started excreting cysts slowly in feces and reached peak by day 7 post infection (PI). Thereafter, the number of excreted cysts started decreasing and the mice became *Giardia* free by day 29 PI and had mean cyst excretion day of 25±2 PI (Figure 2). However, malnourished and renourished-*Giardia*-infected mice (Group V and VI) voided significantly (p<0.001) higher number of cyst. These mice had peak cyst excretion on day 11 PI and became *Giardia* free by day 48 and 42 PI respectively. Interestingly, the pattern of cyst excretion in both malnourished-*Giardia* and renourished-*Giardia*--infected mice (Groups V and VI) was similar to *Giardia*-infected mice (Group IV) but with a higher cyst count and prolonged period of infection (Figure 2).

Trophozoite counts: Oral inoculation of BALB/c mice with *G. duodenalis* trophozoites resulted in the establishment of infection as assessed by the trophozoites count in jejunum. The number of trophozoites increased in all the groups till day 11 PI, thereafter it started decreasing. The mean trophozoite counts were significantly (p<0.001) higher in malnourished-*Giardia*- infected (Group V) and renourished-*Giardia*-infected mice (Group VI) at each point of observation compared with *Giardia*-infected mice (Group IV, Figure 3). However, by day 17 PI, the parasitic load decline in all the groups of mice, except malnourished-*Giardia*-infected mice (Group V, Figure 3), where the decrease in trophozoite number was insignificant.

Total serum protein, albumin and globulin levels: Malnourished-*Giardia*-infected mice (Group V) had significantly lower (p<0.001) levels of total serum proteins, albumin and globulin. Their level decreased further with respect to infection as compared to its controls (Table 1). However, renourished and renourished-*Giardia*-infected mice (Group III &VI) had significantly (p<0.001) higher levels of serum protein, albumin and globulin compared to malnourished mice, but the levels were significantly (p<0.001) lower as compared to *Giardia* infected or control mice (Group I & Group IV, Table 1).

Hemoglobin (Hb): It was observed that malnourished-*Giardia*-infected mice (Group V) had significantly (p<0.001) reduced Hb level as compared with other groups, except the control (Group I, Figure 4).

Differential Leukocyte Count (DLC): DLC was significantly (p<0.001) lower in all the groups of mice compared with control mice (Group I). Malnourished and malnourished-*Giardia*- infected (Group II and V) had significantly (p<0.001) decreased number of DLC

Table 2. Differential Leukocyte Count (DLC) in different groups of mice on different days.

Groups	PI Days	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
Control	7	57±8	37.33±3.0	6.7±1.7	5.6±1.1	0.68±0.3
	11	68.67±11.0	39.3±2.3	6.37±0.85	3.6±1.1	0.6±0.2
	17	57.33±3.0	39.67±7.3	7.8±1.7	6.2±0.9	0.69±0.13
Malnourished	7	28.67±11.67*	29.58±2.8*	5.7±1.4*	1.41±0.6*	0.86±0.04*
	11	40±7.9*	25.98±11.8*	5.73±0.95*	2.0±0.6*	0.27±0.2*
	17	36.33±11.59*	29.8±9.5*	6.6±1.6*	1.54±0.2*	0.49±0.13*
Renourished	7	35.67±11.59*#	33.67±5.1#	6.5±0.8#	4.5±0.5#	0.57±0.05#
	11	53.67±5.1#	35.93±11.5#	7.28±0.7#	3.5±1.2#	0.7±0.1#
	17	52.67±5.5#	37.4±3.6*#	7.9±1.5	5.1±0.6	0.59±0.01#
Giardia	7	42.67±7.76*#	34.4±1.6#	6.07±1.6#	2.6±0.5	0.6±0.2#
	11	56±7.55*#	33.83±4.5#	5.4±0.6$	2.7±0.8*	0.47±0.18*
	17	43.3±7.7*#	34.7±5.0*	5.8±2.2*	3.1±0.75*#	0.57±0.23#
Malnourished Giardia	7	27.67±5.5*$Φ	23.58±6.9*$ Φ	6.1±0.29	1.1±0.9*$	0.7±0.2#$
	11	25±4.3*$ Φ	20.55±3.4*$ Φ	4.06±1.2*$Φ	1.6±1.4*$Φ	0.21±0.2*$
	17	35.33±3.0*$Φ	24.7±7.3*$Φ	4.9±1.7*#$	1.27±1.0*$Φ	0.40±0.17*
Renourished Giardia	7	36.2±12.3*#	30.24±3.5#	5.8±0.81*#	2.76±1.1	0.64±0.3
	11	51.92±5.6*#	26.6±2.3*$Φ	5.2±1.0$	2.3±1.1*	0.43±0.3*
	17	40.96±5.5*#	32.7±3.6*	5.4±1.0*	2.7±1.0*#Φ	0.53±0.53#

Values are mean ±SD,
**p<0.001 v/s Control,*
#p<0.001 v/s malnourished,
$ p<0.001 v/s renourished,
Φp<0.001 v/s Giardia.

with respect to control and renourished mice (Group I & III). However, in all other groups DLC count remained almost the same at each point of observation (Table 2).

Total Leukocyte Count (TLC): Malnourished mice (Group II) had significantly (p<0.001) reduced TLC count compared to control (Group I) or renourished mice (Group III) at each point of observation. However, upon *Giardia* challenge TLC count decreased significantly (p<0.001) in malnourished *Giardia*-infected mice (Group V) compared with controls. The count was the least on day 17 PI, while in renourished mice TLC count increased with time (Group III and VI, Table 3).

Organ mass: Malnourished (Group II) and malnourished-*Giardia*-infected mice (Group V) had a significantly (p<0.001) lower organ mass compared with their controls. The decrease in mass was maximum in malnourished-*Giardia*-infected mice (Group V & Table 4). However, in all other groups, the pattern of weight change remained almost the same with time.

Table 3. Total leukocyte count (TLC) in different groups of mice on different days of post inoculation.

Groups	Day 7 PI	Day 11 PI	Day 17 PI
Control	7616±1. 3	7605±7.5	8363±0.4
Malnourished	4400±1.1*	3201±4.3*	2813±0.6*
Renourished	7876±1.0Ψ	8738±6.6 Ψ	7200±1. 3 Ψ
Giardia	7800±0.6 Ψ	6041±6.3 *,Ψ	7160±1.1*,Ψ
Malnourished *Giardia*	5483±2.2*,Φ,€	2993±4.3*Ψ,Φ,€	1837±0.5*,Ψ,Φ,€
Renourished *Giardia*	6957±0.4*,Ψ,€,≠	7026±7.4*,Ψ, €,≠	7693±0.8*,Ψ, ≠

Values are mean count ±SD,
**p<0.001 v/s Control (day 7, 11,17 PI)*
Ψp<0.001 v/s malnourished (day 7, 11,17 PI),
Φp<0.001 v/s renourished (day 7, 11,17 PI),
€ p<0.001 v/s Giardia (day 7, 11,17 PI),
≠p<0.001 v/s malnourished-Giardia (day 7, 11,17 PI).

4. Discussion

Malnutrition, in its various guises, represents the greatest modifiable threat to global health and survival of human beings. This is especially so among children in the poorest nations of the world, where malnutrition and infections act hand to hand, to create a self- reinforcing downward cycle of tissue depletion and lowered resistance to diseases [4,6,18]. The high prevalence of bacterial and parasitic diseases in developing countries contributes greatly to malnutrition and is the major component of illness and death [19,20,21]. Protein energy malnutrition leads to changes in anthropometric, biochemical and physiological along with alterations in the intestinal morphology [15,16,18,22,23]. Keeping these in mind, the present study was designed to delineate the effect of *Giardia* infection in protein malnourished and renourished mice.

It was observed that malnourished and malnourished mice infected with *Giardia* had significantly less body mass. This may be either due to less food intake (data not shown) or percentage of protein levels. Moreover,

Table 4. Mass of organs in different groups of mice on day 7, 11, 17 PI

Groups	PI Days	Liver(gm)	Spleen(gm)	Small intestine (gm)
Control	7	1.69±0.13	0.38±0.17	2.75±0.08
	11	1.79±0.12	0.54±0.19	1.97±0.03
	17	1.95±0.09	0.69±0.15	2.18±0.14
Malnourished	7	0.77±0.22*	0.11±0.03*	1.08±0.10*
	11	0.66±0.17*	0.08±0.02*	0.98±0.04*
	17	0.51±0.14*	0.07±0.02*	0.90±0.07*
Renourished	7	1.35±0.09*,#	0.26±0.07*,#	1.67±0.27*
	11	1.53±0.14#	0.35±0.11#	1.62±0.08#
	17	1.69±0.15*#	0.45±0.15*#	1.78±0.15*#
Giardia	7	1.42±0.21*,#,$,	0.25±0.03*,#	1.30±0.06*,#
	11	1.65±0.10#	0.21±0.01#	1.71±0.13#
	17	1.76±0.23#	0.4±0.11#	1.92±0.06#
Malnourished *Giardia*	7	0.47±0.16*,#,$,Φ	0.12±0.02*,#,$	1.12±0.17*,#
	11	0.36±0.14*	0.14±0.01*	1.47±0.39*
	17	0.38±0.11*	0.07±0.07*	0.83±0.19*
Renourished *Giardia*	7	1.23±0.09*,#,$,Φ	0.20±0.03*,#	1.01±0.09*,#
	11	1.49±0.15#	0.21±0.06#	1.68±0.39#
	17	1.52±0.05*#	0.34±0.07*#	1.45±0.2*#

Values are mean±SD,
**p<0.001 v/s Control,*
#p<0.001 v/s malnourished,
$ p<0.001 v/s renourished,
Φp<0.001 v/s Giardia

Giardia infection itself leads to maldigestion and malabsorption, thus decreasing the body mass [24-27]. *G duodenalis* trophozoites inoculation in malnourished mice also led to high intensity and prolonged duration of *Giardia* infection compared with renourished or control mice and is in accordance with the earlier study where it has been demonstrated that malnourished Mongolian gerbils infected with *Giardia* had enhanced parasite loads and delay in parasite clearance [28]. This may either be due to impaired immune response of the host or less food intake and protein deficient diet, resulting in prolonged duration of *Giardia* infection.

Giardia trophozoites in small intestine are a recognized marker of *Giardia* infection. In the present study it was observed that in malnourished-*Giardia*-infected animals, trophozoite counts were significantly higher than *Giardia*-infected mice at each point of observation and leading to enhanced severity and duration of *Giardia* infection and is in accordance with earlier studies [29].

Level of blood biochemicals like total serum protein, albumin, globulin, Hb and cell counts e.g. TLC, DLC were all decreased in both malnourished and malnourished mice infected with *Giardia* which may be due to a protein deficient diet that alters the blood and serum biochemical leading to hypoprotienemia and hypoalbuminia [17,13,22]. Study on the organ mass also showed decreased weight in malnourished and malnourished mice infected with *Giardia.* Interestingly, after nourishing such malnourished mice with protein diet, the body mass, blood parameters and mass of all the pivotal organs were improved [23,30].

Based on these observations, it can be proposed that the underlying mechanism for the enhanced susceptibility to the disease and its severity may be due to altered anthropometric and physiological changes in malnourished mice leading to malfunctioning of the body.

In a nut shell, it can be concluded that diet, particularly protein, plays a pivotal role for the proper functioning of the cells, tissues, organs and over all metabolism. The present investigations have indicated that malnourishment increases not only the severity but also the chronicity of *G. duodenalis* infection. Moreover, the mouse model of human giardiasis has the advantage that infection produced in these animals can simulate acute, as well as chronic phase of infection observed in human beings.

5. Acknowlegment

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