

## **Animal Data of Plasma fraction Treatment for Anti-Aging Effect on Rats**

### **1. Objectives of the study:**

- To study the effect of Plasma fraction treatment on age related changes in rats
- Development of antiaging dosage regime to restore the normal ranges of key biomarkers
- To evaluate the safety of the treatment
- To evaluate following parameters in animals
  - Cognitive activities
  - Grip strength
- Biochemical parameters
  - Oxidative stress (GSH, Catalase, MDA and SOD)
  - Rat Nuclear factor erythroid 2-related factor 2 (Nrf2)
  - Histopathology
  - Hematological parameters
  - Inflammatory markers- TNF Alpha and Interleukin 6
  - Weight, food and water intake monitoring

### **2. Treatment and Hypothesis of Study:**

- The treatment is a unique Plasma fraction.
- Majority of age related changes can be reversed using this Plasma fraction treatment.

### **3. Protocol of the Study:**

The details of protocol are mentioned in Table 1

**Table-1: Protocol of the study:**

Sr. No.	Particulars	Description
1	Animals	Sprague Dawley rats
2	Age	Young rats (8 weeks) and Old rats (20 months)
3	Gender	Male
4	Grouping	Young Control 6 Animals
		Old Treated 6 Animals
		Old Vehicle Control 6 Animals
5	Treatment	Plasma fraction
6	Dosing	4 IV injection over 7 days
7	Duration of treatment	7 Days
8	Evaluation Time Points	Initial 0,4 ,8,15 and 30 days

#### **4. Evaluation Parameters:**

1. Body Weight
2. Barnes Maze Learning Ability
3. Grip Strength
4. Hematological Parameters
5. Biochemical Parameters
6. Oxidative stress biomarkers
  - (i) *Reduced glutathione (GSH)*
  - (ii) *Catalase activity*
  - (iii) *Superoxide dismutase (SOD) activity*
  - (iv) *Lipid peroxidation (LPO) (malondialdehyde (MDA))*
7. Rat Nuclear factor erythroid 2-related factor 2 (Nrf2)
8. Anti-inflammatory Markers
  - (i) Interleukin 6 (IL-6)
  - (ii) Tumor Necrosis Factor (TNF) Alpha
9. Histopathology of Vital Organs

## 5. Procedure:

- a. The protocol of study is mentioned in Table 1. The dose of Plasma fraction treatment was prepared and injected intravenously using saline as vehicle.
- b. The calculated doses were administered intravenously to the Animals of Old treated group every alternate day up to 8 days.(4 Injections)
- c. Same amount of saline is used as placebo and administered to the animals of Old control group.
- d. Blood samples were withdrawn by retro orbital plexus at 0, 4, 8, 15 and 30<sup>th</sup> days of treatment for hematological parameters
- e. Serum was separated from the blood samples of each animal and evaluated for biochemical parameters.
- f. Plasma was separated from the blood samples of each animal and evaluated for inflammatory markers i.e. TNF alpha and IL6.
- g. All animals from each group were sacrificed at 30<sup>th</sup> day of treatment and vital organs (Brain, Heart, Lung, Liver, Spleen, Kidney and Testis) of these animals were harvested and taken for histopathology and Immunohistochemistry.
- h. Learning ability of animals was evaluated using Barnes Maze apparatus at each time points after training of one week.
- i. Muscle strength of animals were evaluated using Grip strength meter at each time points.
- j. Body weight, food and water intake of the animals were monitored at each time points

## 6. Results and Discussion:

### 6.1. Body Weight:

Body weight of the old animals and young animals was increased over a period of 30<sup>th</sup> days. There was no change observed in the food intake however an increase in water intake was evident in old treated animals group.

Table-2

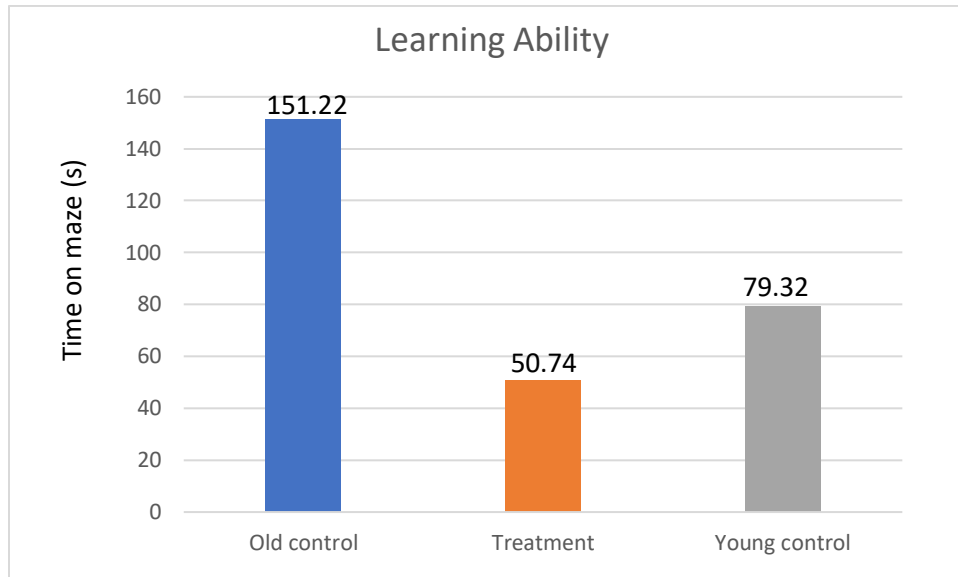
Group	Old control	Old Treated	Young Control
0 Day	508.00	514.33	257.63
4 Day	508.33	514.33	258.32
8 Day	512.17	517.50	260.32
15 Day	512.67	517.33	262.25
30 Day	514.67	518.33	279.25



Figure 2.

## 6.2. Learning Ability:

A Barnes maze apparatus used to determine the learning ability of the animals upon treatment. The data represented in figure 3 shows % decrease in time spent on maze by rats. The data of day 1 and day 7 was compared to determine the learning ability. The data shows decrease in the learning ability in the animals of old control group however it is increased in the group of old treated animals. The data of treated old and young group is comparable.



**Figure 3: Learning ability using Barnes maze (at 30<sup>th</sup> Days).**

### 6.3. Muscle Strength:

Grip strength meter was used to determine the muscle strength of animals the data is represented in Figure 4. The data shows significant increase in muscle strength in the treated old group compared to old control group treatment. The data of treated old group is comparable to young animal's group.

Table-3

Group	Old control	Treatment	Young Control
0 Day	6.10	6.31	10.25
4 Day	6.24	8.25	10.84
8 Day	6.01	11.38	10.55
15 Day	6.00	11.55	11.35
30 Day	5.78	11.74	12.01

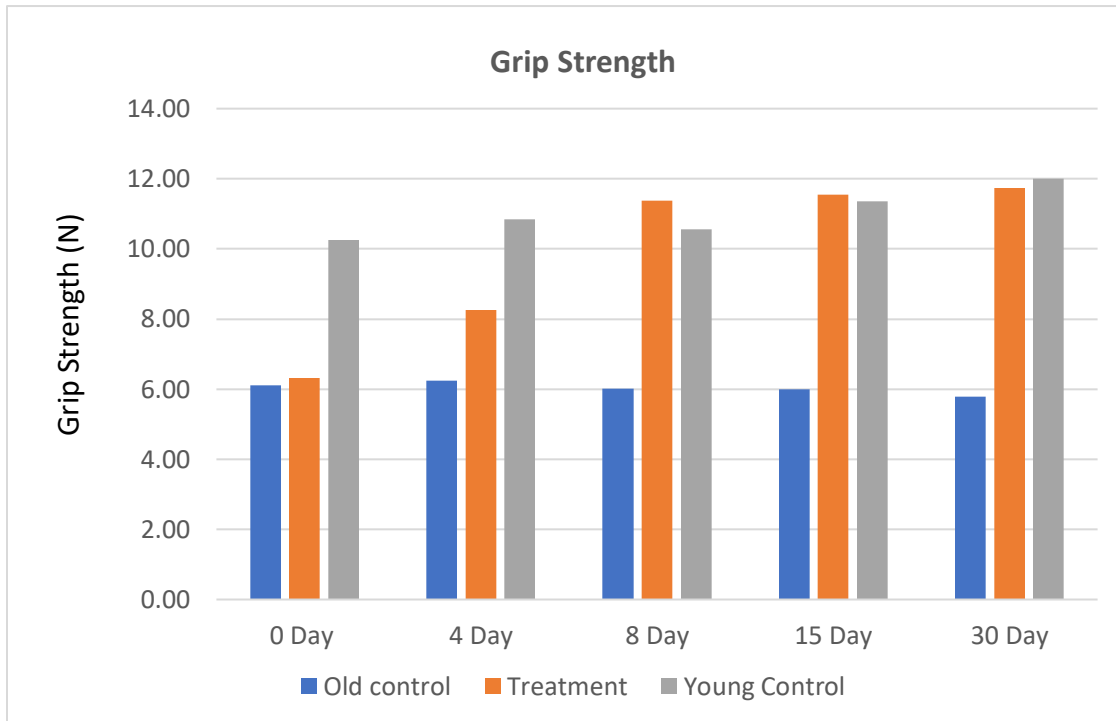


Figure 4: Muscle Strength of animals.

The Table 3 shows significant difference in the grip strength of old control and treated old groups. However, there is no significant difference in treated old and young control.

#### 6.4. Hematological Parameters:

Data represented in Table No. 4,5 and 6 does not show markable difference in the determined values among groups.

#### 0 Day

	Samplecode	Hb gm %	RBC x 10 <sup>6</sup> /cmm	WBC X 10 <sup>3</sup> /cmm	PLT X 10 <sup>5</sup> /cmm	PCV %	MCV fl	MCH pg	MCHC gm/dl	N %	E %	L %	M %
Old control	1	14.9	7.27	9.8	818	38.6	53.1	20.5	38.6	39	04	57	00
	2	14.9	7.32	12.0	822	38.9	53.1	20.4	38.3	42	00	57	01
	3	14.2	6.52	8.2	768	36.9	56.6	21.8	38.5	41	02	56	01
	4	14.6	6.79	11.2	784	38.4	56.6	21.5	38.0	44	01	54	01
	5	13.2	6.93	11.4	771	34.2	49.4	19.0	38.6	51	02	47	00
	6	13.4	7.08	14.8	771	35.1	49.4	18.9	38.2	32	00	68	00
Treatment	1	14.2	6.68	12.2	773	36.3	52.9	20.7	39.1	33	06	60	01
	2	14.0	6.83	8.8	732	36.2	53.0	20.5	38.7	44	02	53	01
	3	13.1	6.94	13.0	755	33.0	47.6	18.9	39.7	51	02	47	00
	4	13.0	7.10	8.1	740	33.7	47.5	18.3	38.6	48	03	49	00
	5	13.7	7.04	12.3	807	34.3	48.7	19.5	39.9	32	01	67	00
	6	14.0	7.16	11.1	798	35.5	49.6	19.6	39.4	49	04	46	01

Table 4: Hematological evaluation of animals at 0 Day

#### 15 days

	Samplecode	Hb gm %	RBC x 10 <sup>6</sup> /cmm	WBC X 10 <sup>3</sup> /cmm	PLT X 10 <sup>5</sup> /cmm	PCV %	MCV fl	MCH pg	MCHC gm/dl	N %	E %	L %	M %
Old control	1	12.1	6.26	12.9	993	32.9	52.6	19.3	36.8	28	01	70	01
	2	12.5	6.55	12.9	949	33.9	51.8	19.1	36.9	42	10	47	01
	3	11.8	6.06	7.8	978	31.5	52.0	19.5	37.5	46	01	53	00
	4	10.2	5.40	10.2	748	28.5	52.8	18.9	35.8	51	01	47	01
	5	13.1	6.66	10.5	890	34.2	51.4	19.7	38.3	31	02	66	01
	6	11.2	5.51	7.9	832	30.4	55.2	20.3	36.8	49	03	47	01
Treatment	1	13.8	7.09	8.9	776	34.9	49.2	19.5	39.5	37	00	63	00

	2	10.9	5.42	9.4	927	29.3	54.1	20.1	37.2	51	01	47	01
	3	10.6	5.47	7.6	930	29.6	54.1	19.4	35.8	27	02	71	00
	4	10.4	5.25	5.0	931	28.2	53.7	19.8	36.9	33	00	66	01
	5	11.6	6.47	11.4	1082	33.6	52.0	17.9	34.5	28	04	66	02
	6	12.6	6.82	9.8	1099	35.1	51.4	18.4	35.8	30	02	67	01

**Table 5: Hematological evaluation of animals at 15<sup>th</sup> Day**

**30<sup>th</sup> days**

	Samplecode	Hb gm %	RBC x 10 <sup>6</sup> /cmm	WBC X 10 <sup>3</sup> /cmm	PLT X 10 <sup>5</sup> /cmm	PCV %	MCV fl	MCH pg	MCHC gm/dl	N %	E %	L %	M %
<b>Old control</b>	1	13.1	7.31	15.2	1101	38.0	52.1	17.9	34.4	31	01	68	00
	2	14.3	7.28	12.7	1113	40.5	55.7	19.6	35.3	28	03	68	01
	3	15.4	7.86	14.2	718	43.3	55.1	19.5	35.5	42	00	57	01
	4	13.9	7.65	13.5	759	39.4	51.6	18.1	35.2	31	05	63	01
	5	15.3	7.94	10.5	516	43.4	54.7	19.2	35.2	22	02	76	00
	6	13.9	7.79	8.2	750	40.1	51.6	17.8	34.6	42	02	55	01
<b>Treatment</b>	1	14.1	7.56	11.8	969	39.4	52.2	18.6	35.7	38	06	56	00
	2	13.9	7.29	9.7	1016	38.1	52.3	19.0	36.4	33	04	62	01
	3	13.3	6.98	8.5	806	38.2	52.1	19.2	31.0	43	01	54	02
	4	13.4	7.01	9.8	735	38.6	52.6	19.6	31.3	30	02	67	01
	5	14.4	7.59	14.4	943	40.1	52.9	18.9	35.9	29	08	63	00
	6	13.6	7.48	9.4	779	39.3	52.6	19.3	36.2	21	02	75	02

**Table 6: Hematological evaluation of animals at 1 month**



### 6.5. Biochemical Parameters:

Data represented in Table 7 shows changes in the biochemical parameters of various organs. The liver function tests and kidney function tests are found to be better in case of treated old group compared to old vehicle control group after 8 days of treatment. The results shown in blue demonstrates significant difference between old control and old treated groups however no significant difference between young control and old treated.

Sr.No.	Parameter		Old control	Treatment	Young Control
1	Total protein (g/dl)	0 Day	8.08	8.79	4.48
		4 Day	8.39	6.99	4.54
		8 Day	8.58	5.77	4.58
		15 Day	8.82	5.91	4.67
		30 Day	9.32	6.10	4.78
2	Total Bilirubin (mg/dL)	0 Day	0.61	0.52	0.24
		4 Day	0.60	0.49	0.25
		8 Day	0.61	0.31	0.25
		15 Day	0.63	0.34	0.27
		30 Day	0.66	0.44	0.28
3	Direct Bilirubin (mg/dL)	0 Day	0.327	0.332	0.127
		4 Day	0.330	0.285	0.133
		8 Day	0.353	0.238	0.143
		15 Day	0.372	0.248	0.155
		30 Day	0.398	0.262	0.168
4	Glucose (mg/dL)	0 Day	172.61	171.22	68.71
		4 Day	172.80	161.40	69.70
		8 Day	173.67	152.85	71.01
		15 Day	174.45	139.90	72.36
		30 Day	175.96	117.81	75.03
5	Triglyceride (mg/dL)	0 Day	63.79	62.83	34.54
		4 Day	64.98	41.09	35.04
		8 Day	66.20	38.42	35.71
		15 Day	67.25	39.76	36.86
		30 Day	69.41	41.54	37.56
6	HDL (mg/dL)	0 Day	191.34	194.86	380.03
		4 Day	191.11	201.41	380.06

		<b>8 Day</b>	191.23	202.04	380.10
		<b>15 Day</b>	191.61	204.02	380.37
		<b>30 Day</b>	<b>190.90</b>	<b>210.58</b>	<b>381.17</b>
7	<b>Cholesterol (mg/dL)</b>	<b>0 Day</b>	46.12	45.01	18.05
		<b>4 Day</b>	45.99	32.07	18.15
		<b>8 Day</b>	46.18	24.15	18.40
		<b>15 Day</b>	46.99	26.19	18.73
		<b>30 Day</b>	<b>48.17</b>	<b>22.35</b>	<b>19.23</b>
8	<b>Albumin (g/dl)</b>	<b>0 Day</b>	1.83	1.84	3.07
		<b>4 Day</b>	1.83	2.13	3.14
		<b>8 Day</b>	1.82	2.48	3.38
		<b>15 Day</b>	1.77	2.72	3.54
		<b>30 Day</b>	<b>1.71</b>	<b>3.12</b>	<b>3.87</b>
9	<b>Creatinine (mg/dL)</b>	<b>0 Day</b>	0.80	0.78	0.25
		<b>4 Day</b>	0.81	0.53	0.26
		<b>8 Day</b>	0.82	0.51	0.26
		<b>15 Day</b>	0.82	0.42	0.27
		<b>30 Day</b>	<b>0.84</b>	<b>0.37</b>	<b>0.34</b>
10	<b>BUN (mg/dL)</b>	<b>0 Day</b>	14.55	15.25	4.39
		<b>4 Day</b>	14.22	12.34	4.41
		<b>8 Day</b>	14.23	10.42	4.46
		<b>15 Day</b>	14.25	8.78	4.51
		<b>30 Day</b>	<b>14.36</b>	<b>8.22</b>	<b>4.57</b>
11	<b>SGPT (IU/L)</b>	<b>0 Day</b>	41.44	40.80	15.21
		<b>4 Day</b>	42.38	38.49	15.25
		<b>8 Day</b>	43.08	32.86	15.70
		<b>15 Day</b>	46.55	30.82	16.11
		<b>30 Day</b>	<b>48.43</b>	<b>23.58</b>	<b>17.78</b>
12	<b>SGOT (IU/L)</b>	<b>0 Day</b>	101.90	103.71	25.76
		<b>4 Day</b>	102.61	87.69	26.35
		<b>8 Day</b>	103.37	69.69	27.38
		<b>15 Day</b>	104.22	55.30	29.78
		<b>30 Day</b>	<b>108.57</b>	<b>46.08</b>	<b>32.74</b>

**Table 7: Biochemical evaluation**

6.6. Alterations in oxidative stress biomarkers

(i) *Reduced glutathione (GSH)* :

Table-8

	Brain	Heart	Lung	Liver
Old Control	12.6852	28.0231	9.33875	16.3878
Old Treatment	35.4516	58.176	23.9225	45.9731
Young Control	40.1528	73.8892	30.5975	66.1527

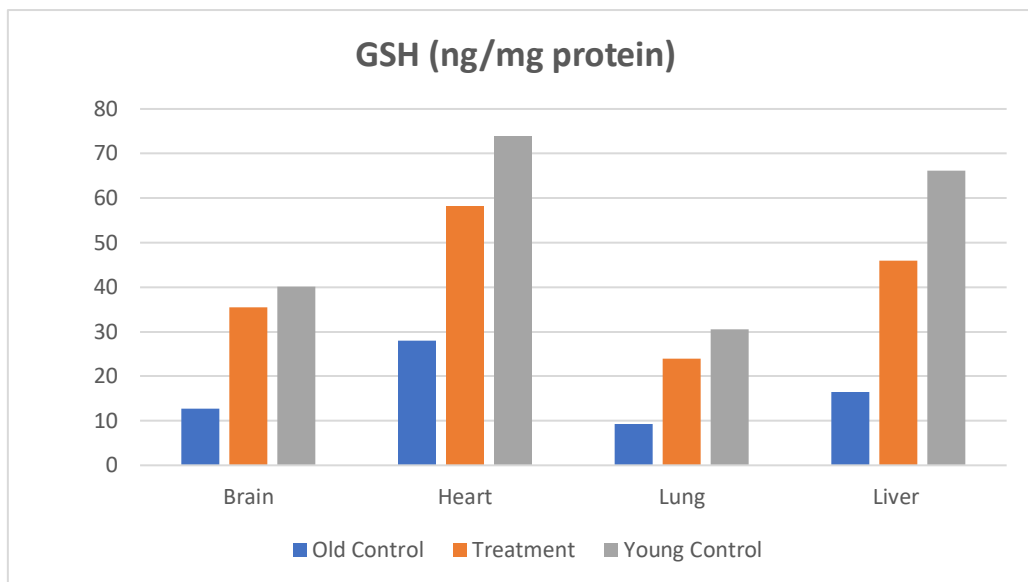
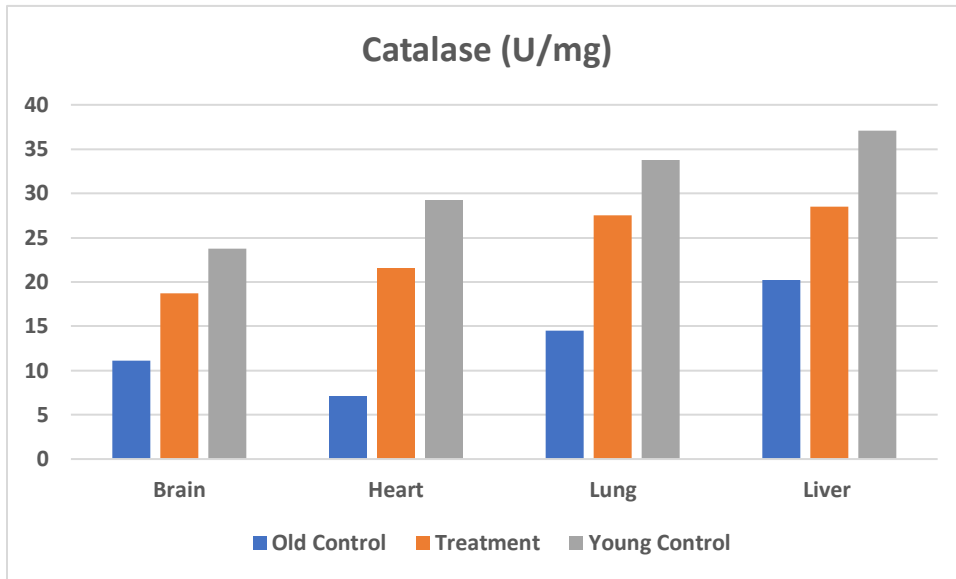


Figure 5: *Reduced glutathione (GSH)*.

**(ii) Catalase activity**

**Table-9**

	<b>Brain</b>	<b>Heart</b>	<b>Lung</b>	<b>Liver</b>
<b>Old Control</b>	11.0935	7.09036	14.5049	20.2093
<b>Treatment</b>	18.7342	21.5619	27.5134	28.4885
<b>Young Control</b>	23.7575	29.2787	33.7523	37.0732

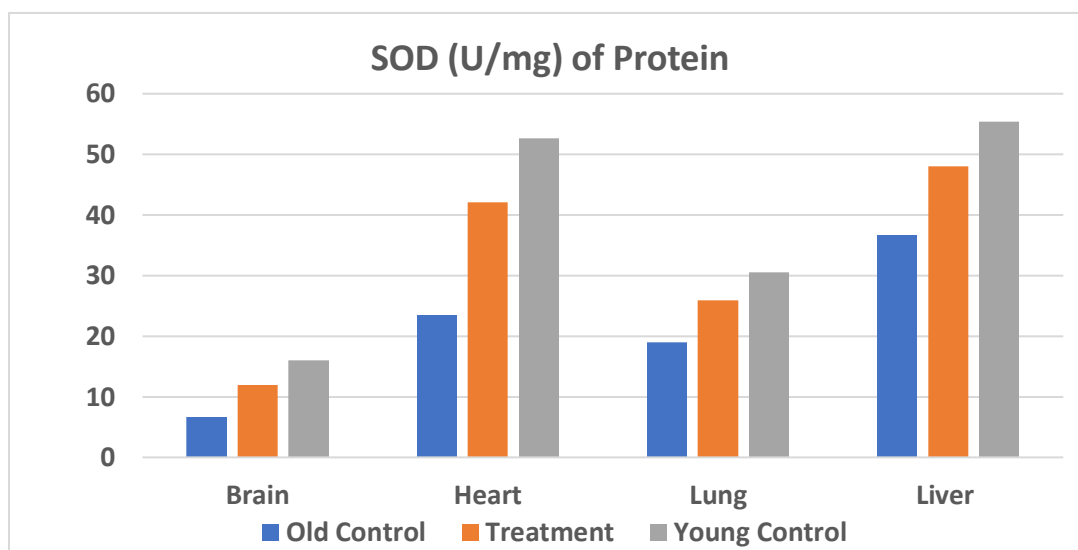


**Figure 6: Catalase.**

**(iii) Superoxide dismutase (SOD) activity**

**Table-10**

	<b>Brain</b>	<b>Heart</b>	<b>Lung</b>	<b>Liver</b>
<b>Old Control</b>	6.6062	23.4765	18.9899	36.7429
<b>Treatment</b>	11.9003	42.0798	25.9351	47.9919
<b>Young Control</b>	16.0155	52.6473	30.5124	55.3808

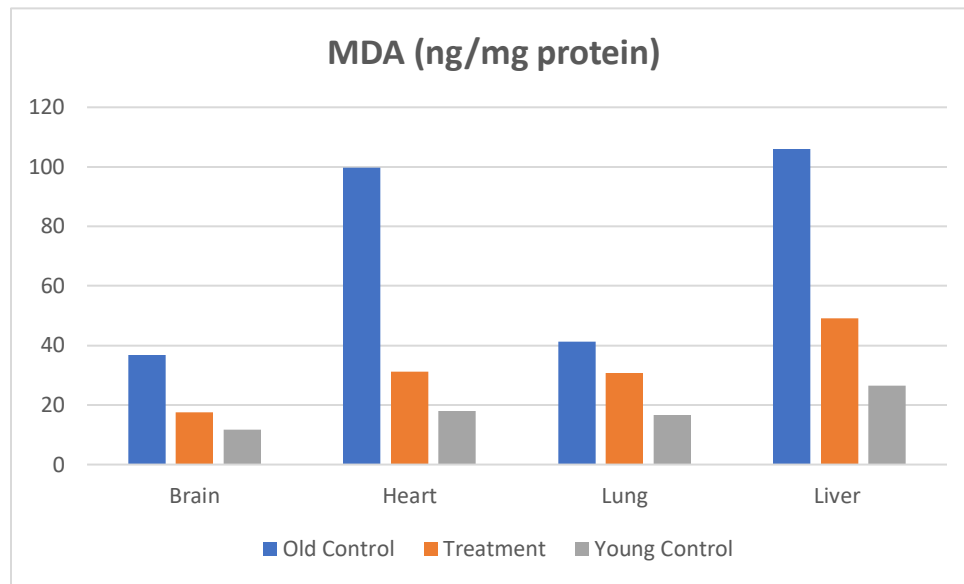


**Figure 7: Superoxide dismutase (SOD)**

**(iv) Lipid peroxidation (LPO) (malondialdehyde (MDA))**

**Table-10**

	<b>Brain</b>	<b>Heart</b>	<b>Lung</b>	<b>Liver</b>
<b>Old Control</b>	36.9041	99.8131	41.3109	106.054
<b>Treatment</b>	17.5007	31.1292	30.6645	49.1078
<b>Young Control</b>	11.6804	18.0082	16.762	26.4917



**Figure 8: Malondialdehyde (MDA)**

### 6.7. Rat Nuclear factor erythroid 2-related factor 2 (Nrf2)

Table-11

	Brain	Heart	Lung	Liver
Old Control	6.7	9.13182	13.9614	8.84773
Treatment	8.325	17.9386	14.5636	18.6091
Young Control	10.0636	20.8477	18.6091	17.0068

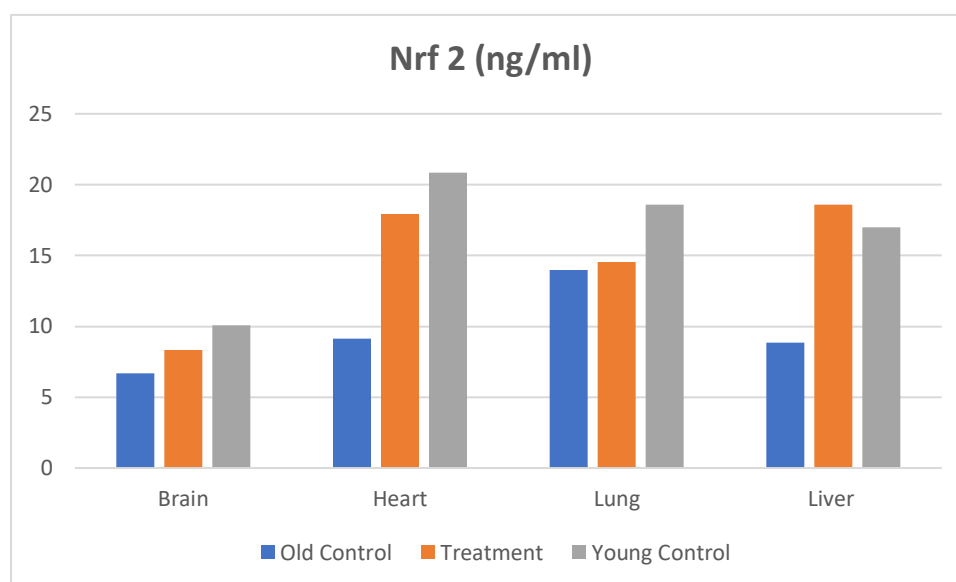


Figure 9: Nrf 2

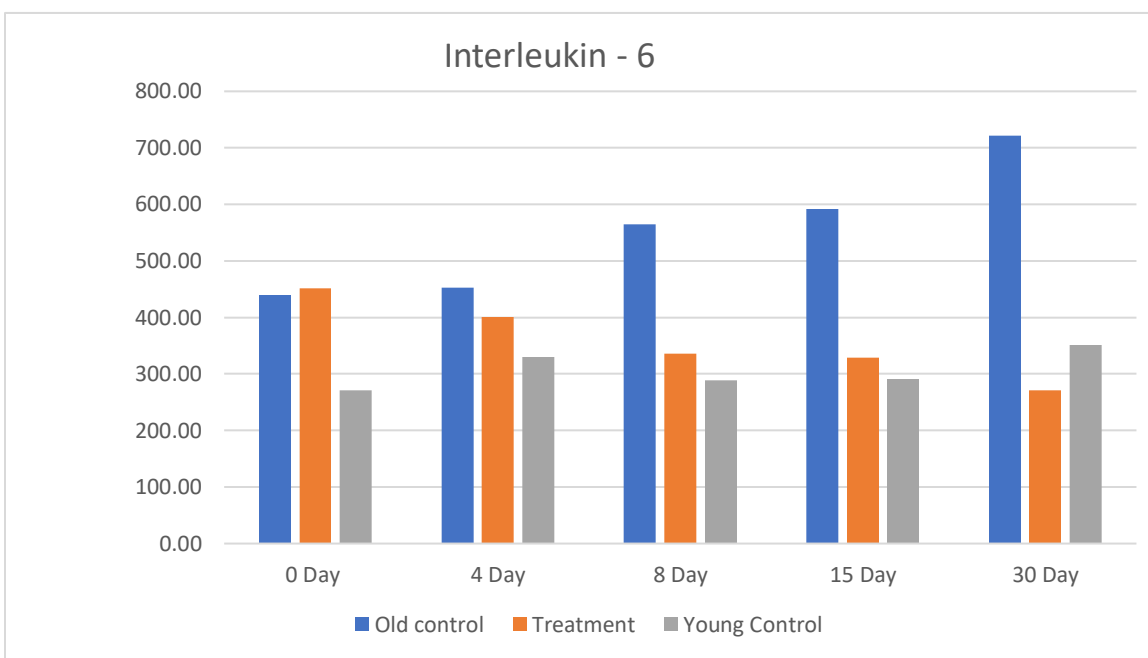
## 6.8. Anti-inflammatory Markers:

### 1. Interleukin-6:

The data represented in Figure- 10 shows remarkable decrease in IL-6 concentration in the treated old group compared to old control group. The data of treated old group is comparable to young animal's group.

**Table-12**

Group	Old control	Treatment	Young Control
<b>0 Day</b>	439.67	451.33	270.78
<b>4 Day</b>	453.00	401.33	330.22
<b>8 Day</b>	564.67	335.78	289.11
<b>15 Day</b>	591.89	329.11	290.78
<b>30 Day</b>	721.89	270.78	350.78



**Figure 10: Concentration of Interleukin-6 .**

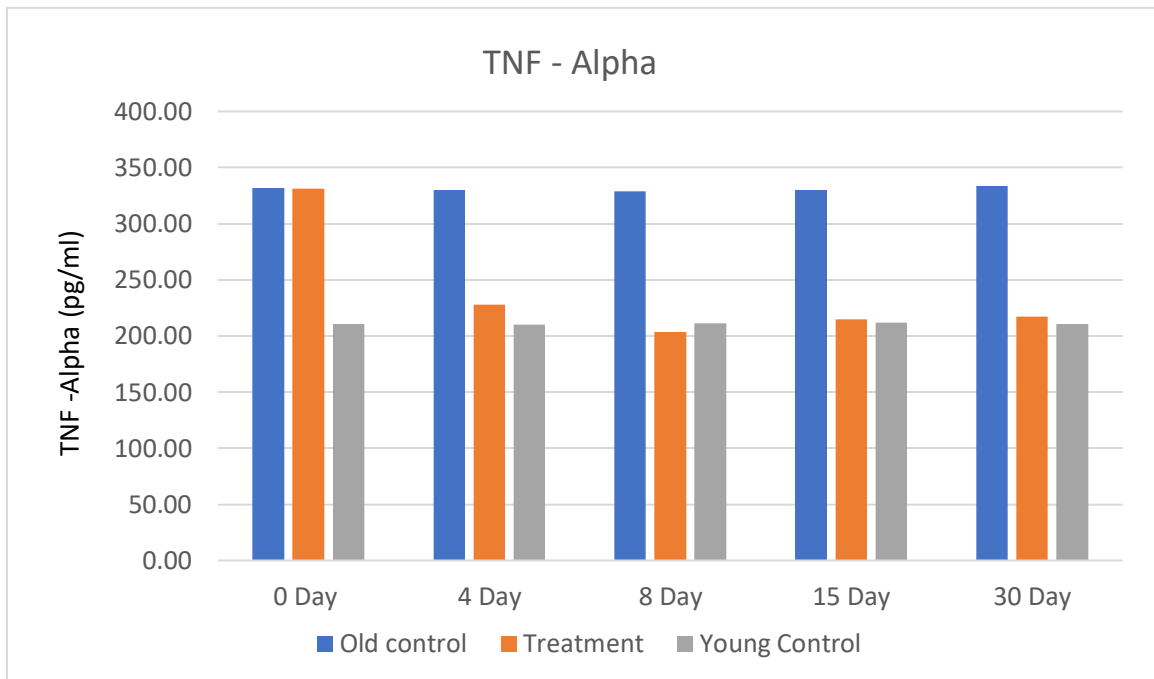


## 2. Tumor Necrosis Factor (TNF) Alpha:

The data represented in Figure- 2 shows significant decrease in TNF Alpha concentration in the treated old group compared to old control group after 30 days of treatment.

**Table-13**

Group	Old control	Treatment	Young Control
<b>0 Day</b>	331.71	331.29	210.46
<b>4 Day</b>	330.25	227.96	210.25
<b>8 Day</b>	328.58	203.58	211.50
<b>15 Day</b>	329.83	214.63	211.71
<b>30 Day</b>	333.38	217.13	210.88



**Figure 11: Concentration of TNF Alpha.**

## 6.9. Histopathology of Vital Organs :

**Histopathological evaluation of vital organs does not revealed any major abnormality please refer separate report of histopathology.**

### 1. Conclusion:

- Plasma fraction treatment showed significant improvement in the old animals
- Plasma fraction treatment can reverse the age related changes
- It could be helpful in preventing the age related and lifestyle disorders
- This treatment is safe as no abnormality observed in animals

### 2. Future Plan:

- Development of suitable dosage form
- Stability study of the formulations
- Patent application- in progress
- Human clinical trials- under discussion
- Regulatory approvals
- Marketing strategy finalization
- Commercialization

