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ULTRA DILUTED ARSENIC-INDUCED ALTERED CYTOKINE GENE EXPRESSIONS IN EMBRYONATED EGGS CHALLENGED WITH SARS-COV-2 SPIKE PROTEIN RBD ANTIGEN

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Keywords:

SARS-CoV-2, RBD, Arsenic 6C, *Gallus gallus* embryo, IL-10, hepato-protective action

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ABSTRACT: Introduction / Objective: In this study, we aimed to elucidate the action of ultra-diluted (potency 6CH as per homeopathic pharmacopeia) ethanolic extract of *Arsenicum album* (Arsenic 6CH) on cytokine gene expression changes along with alteration of hepatic histology in *Gallus gallus* embryo (embryonated egg) challenged with the spike protein (S protein RBD of Delta SARS-CoV-2). **Methods:** Allantoic fluid was collected during harvesting of the inoculated eggs after antigenic challenge. The gene expression analysis of interferons (IFN α, IFN β, IFN γ); transforming growth factor-beta 1 (TGF β1); interleukins (IL-6, IL-8, IL-10, IL-1β) were studied in real-time PCR. Pre- and post-treatment sets were separately studied. Hepatic tissue of the embryos was collected for histological study. **Results:** The most prominent changes were found with IL-10 expression, which was significantly increased directly by Arsenic 6CH; in pre-and post-treatment experimental sets, its level was further increased, while in control sets, such changes were not delineated. Some changes were found in relation to INF β, IL-8, IL-1β, and TGF β1 in alcohol 6CH (potentized vehicle alcohol as per homeopathic pharmacopoeia), and there was no significant change in other experimental sets. Histological studies of the liver indicated definite protective action of Arsenic 6CH from hepatic pathological changes in both pre and post-treatment experimental sets. **Conclusion:** Our study indicates enhancement of beneficial anti-inflammatory cytokines with hepato-protective action by Arsenic 6CH in *Gallus gallus* embryo challenged with Delta SARS-CoV-2 spike protein RBD antigen inducing pathological changes.

INTRODUCTION: The severe acute respiratory syndrome (SARS) coronavirus-2 is a novel strain of coronavirus and it belongs to the family Coronaviridae [1]. This strain is the causative agent for severe acute respiratory infections that originated in Wuhan, China [1]. The disease has been named corona-virus disease -19 or COVID-19 [1].

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This strain of the virus is highly homologous to the original strain SARS-CoV that demonstrated pandemic respiratory infection in the year 2002 – 2003 [2]. The cause of the rapid spread of the disease is the direct transmission from human to human which resulted in the community spread with the inclusion of about two hundred countries throughout the globe [1, 2].

The viral transmission occurs with the aid of respiratory droplets and aerosols. After the virus enters our body, it binds with the receptors of host cells and, after that, causes membrane fusion or endocytosis [2, 3]. The virus consists of four structural proteins - spike (S), membrane (M), envelop (E),

and nucleocapsid (N) proteins [3, 4, 5]. Thorough studies have revealed that ACE 2 is the primary functional receptor with which the virus SARS-CoV- 2 binds and then two-step protease cleavage occurs for its activation [4]. Following the entry of the virus within the host cells, the contents of the virus get released. Inside the cells, the virus undergoes replication with the aid of RNA polymerase activity in a complex pathway as reported by several researchers [5 - 16].

Homeopathy, a branch of alternative medicine, is based on the principle of 'like cures like' [17]. Science heals the disease by inducing the healing response, and this happens with the administration of drugs that causes similar disease-like symptoms within the individuals. Ministry of Health & Family Welfare, Government of India, has released some prophylactic measures for the healthy population against COVID-19 (Guidelines for Homoeopathy Practitioners for Prophylaxis and Symptomatic Management of COVID-19 Patients in Home Isolation by Ministry of Ayush, Government of India, 2020).

Within this guideline, the Scientific Advisory Board of the Central Council for Research in Homoeopathy (CCRH) has stressed the usage of *Arsenicum album* (ultra diluted arsenic trioxide), which would act as an immune-modulatory agent for the prophylaxis against COVID -19 infection. It is well known that homeopathy is the branch of science that has offered significant help to the common people with respect to the maintenance of health and the treatment of a broad range of illnesses.

This mode of treatment is acceptable to the common people because it is quite cheaper than the conventional treatment and also because of its almost negligible side effects [18]. In this regard, Mathie *et al.*, in 2013, depicted within their study paper on 1126 patients suffering from swine flu (2009 pandemic of A/H1N1 influenza) that *Arsenicum album* was found to be the most frequently prescribed medication in comparison to other medications (n = 28; 28.0%) [19]. The antiviral activity of arsenic trioxide (As_2O_3) has also been reported at a sub-micromolar concentration level against the Hepatitis C virus (HCV). The action mechanism was found to block the replication of the virus. The study reported that the 50% effective concentration or the EC_{50} value of the arsenic trioxide against the viral replication of HCV was 0.35 µM and the compound As_2O_3 did not reveal any cellular toxicity at that particular concentration. The study also revealed the synergistic effect of arsenic trioxide with alpha-interferon against the HCV virus [20].

Thus, in the present study, we explored to elucidate the action of the ultra-diluted ethanolic extract of *Arsenicum album* of homeopathic potency 6CH, against the induced pathological changes by the spike protein RBD of Delta SARS-CoV-2 in embryonated chick egg model (*Gallus gallus domesticus*).

MATERIAL AND METHODS:

The Spike Protein and the Medicine: Spike protein (S) receptor-binding domain (RBD) antigen (L452R, E484Q) of SARS-CoV-2 (B.1.617, Delta variant) was procured from Abclonal, USA (Product code: WH192258, Lot: 9621050601, Cat. No. RPO2266). Ultra-diluted ethanolic extract of Arsenicum album (6CH potency dilution as per homeopathic pharmacopeia containing material at attogram level) was procured from a reputed government-approved alternative medicine producing industry. "HAPCO, India" for the experimental purpose.

Egg Procurement and Inoculation Technique: Fertilized chick eggs (*Gallus gallus domesticus*) of 14th day old were procured from the State Poultry Farm, Kolkata. The surfaces of the eggs were cleaned with distilled water, and a marker pen marked the air sacs after candling the eggs.

The candling also helps to differentiate the dead and live embryonated eggs. The dead chick embryos were not included in the experiment. The egg shells over the air sacs were disinfected with 70% ethanol and povidone-iodine solution (10% w/v solution) and then small punctures were made at the center of air sacs with the aid of a sterile needle [21]. Identification marks were given in different experimental sets (specified below). Then the desired materials of volume 100 µL were injected with 1ml syringe via the amniotic route of the eggs. Following seven experimental sets were arranged for the study:

1. Control (14^{th}-day embryonated eggs).
2. Alcohol control (14^{th} day embryonated eggs challenged with 70% v/v molecular grade ethanol).
3. Potentized alcohol 6CH control (procured from HAPCO, embryonated eggs challenged with Alcohol 6CH).
4. Medicine control (embryonated eggs challenged with Arsenic 6CH).
5. Antigen control (embryonated eggs challenged with original spike protein (S) RBD antigen at 10 µg/mL concentration dissolved in Phosphate buffer saline).
6. To observe the pre-treatment action of the medicine against the S protein-induced pathological changes, ultra-diluted *Arsenic album* 6CH was inoculated first, followed by S antigen after one hour of incubation.
7. To observe the post-treatment action of the medicine against the S protein-induced pathological changes, the S antigen was inoculated first, followed by ultra-diluted *Arsenic album* 6CH after one hour of incubation.

After inoculation procedures, the sites of punctures were sealed using a sterile sticker, and all the inoculated eggs were incubated at 38º C at 60% humidity for 48 h.

Egg Harvesting and Collection of Allantoic Fluid and Hepatic Tissue: All the eggs were kept in the refrigerator at 4 ºC for two hours prior to harvesting to collect allantoic fluid and hepatic tissue samples analysis.

The eggs were cut opened using sterile scissors and forceps for the collection of 5-10 mL of allantoic fluid, and then liver tissue samples were collected and stored in 10% formol-saline for histological study. The fluids were collected in sterile containers and stored at -80 º C for further study. The gross general appearances of the embryos were also observed and recorded [21].

Molecular Biology Study: The total RNA was extracted from the allantoic fluid using the RNA is plus (trizol) method from all the fluid samples. The RNA was quantified using a UV-Vis spectrophotometer (Agilent, USA) by A260/A280 ratio. cDNA was synthesized with the purified RNA using a cDNA synthesis kit (iscript Reverse Transcript Transcriptase, Bio-Rad, USA) in a conventional thermal cycler (T 100, Bio-Rad, USA). Semi-quantitative gene expression analysis was carried out of 8 cytokines genes, namely chicken Interferons (chIFN) α, β, γ; chicken Interleukins – chIL-6, chIL-8, chIL-10, chIL-1 β; chicken Transforming Growth Factor (chTGF) β1 with respect to β-actin (control housekeeping gene). For the RT PCR analysis, 2 µL of cDNA and 18µL of Taq universal cyber green supermix (Bio-Rad, USA) were mixed and analyzed with CFX-96 model (Bio-Rad, USA) RT-PCR instrument following standard protocol [22].

Histological Study: After fixation in formol-saline, the paraffin blocks were prepared with the tissue samples, and then 3 – 5 µm thick sections were made in microtome. After that haematoxylin and eosin staining (H & E staining) was done following the standard guidelines [23].

Statistical Analysis: Statistical analysis was conducted using Python software for correlation values, regression equations, and analysis of variance.

RESULTS: Among different cytokines, the most prominent changes were found with an expression of IL-10 gene. Expression of this anti-inflammatory cytokine was significantly increased directly by Arsenic 6CH; again, in pre and post-treatment experimental sets its level was further increased. However, in control sets, such changes were not observed.

Some cytokines, namely INF β, IL-8, IL-1β and TGF β1 showed a uniform pattern with significantly increased levels with alcohol 6CH without any significant change in all other experimental sets.

Although IL-6 and INF α (except non potentized alcohol experimental set) showed increased expressions in all experimental sets, the increased levels were comparable to the experimental set of alcohol 6CH. Details of all cytokine changes are given in the graphs **Fig.1-8.**

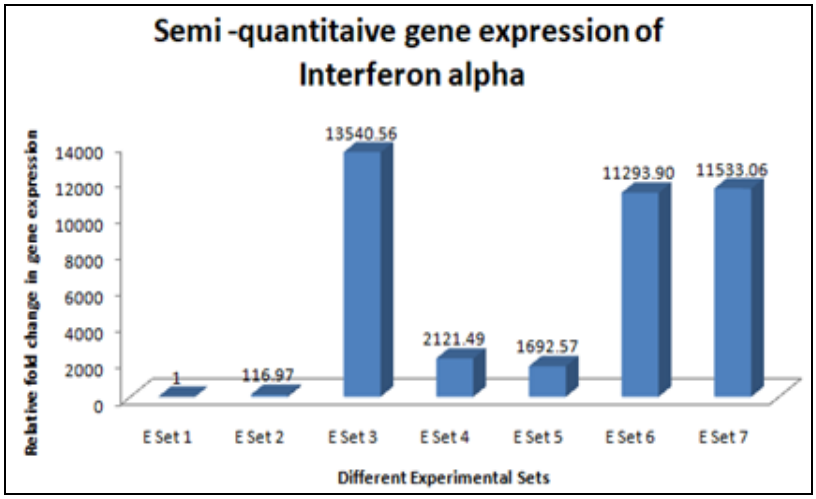


FIG. 1: CHANGES OF IFN ALPHA ARE MAINLY BY ALCOHOL 6CH, WHICH IS THE VEHICLE OF MEDICINE ARSENIC 6CH

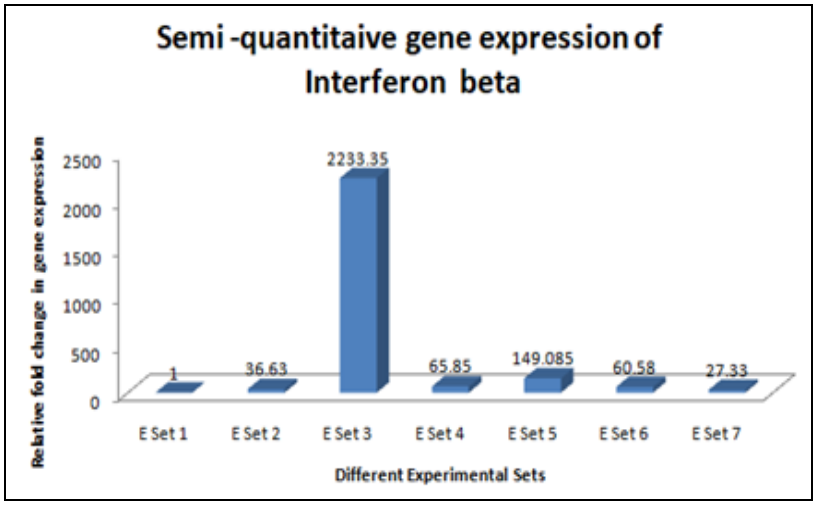


FIG. 2: CHANGES IN IFN BETA GENE EXPRESSION ARE NOT SO PROMINENT, AND IN MOST OF THE CASES, ARSENIC 6CH, EITHER DIRECTLY OR IN PRE-TREATMENT AND POST-TREATMENT ROLES, DECREASED THE SIGNIFICANT EXPRESSION OF IFN BETA GENE EXPRESSION, WHICH WAS FOUND WITH ALCOHOL 6CH

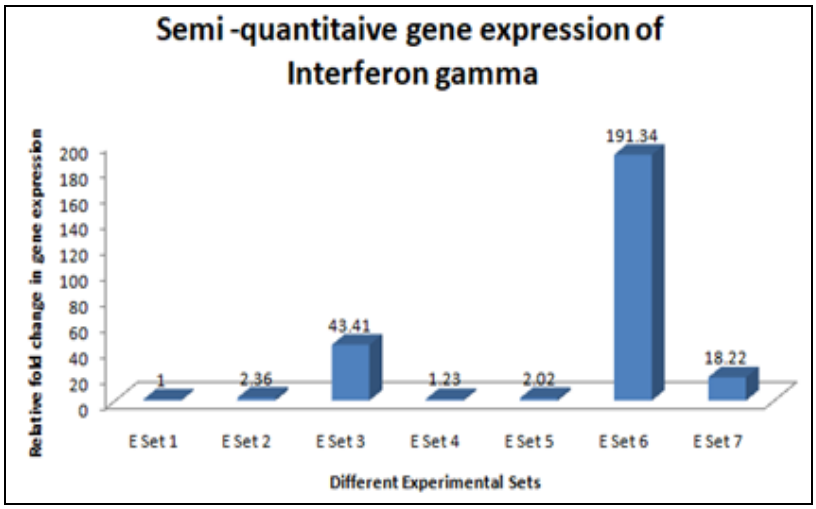


FIG. 3: IN CASE OF IFN GAMMA, ARSENIC 6CH DECREASES THE EXPRESSION OF INTERFERON-GAMMA THAN THE CONTROLS, BUT IF IT IS USED AS A PRE-TREATMENT AGENT, THEN, IT UP-REGULATES THE IFN GAMMA GENE SIGNIFICANTLY, ABOUT 200 TIMES MORE THAN THE DIRECT ACTION OF ARSENIC 6CH. IN THE POST-TREATMENT ALSO IT UP-REGULATES THIS GENE MORE THAN 15 TIMES

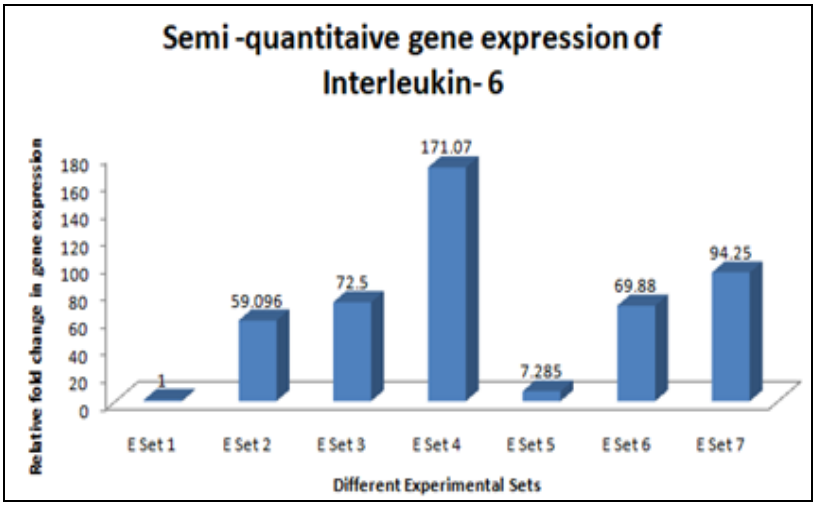


FIG. 4: WHEN WE ARE CONSIDERING THE IL-6 EXPRESSION, THEN AGAIN, IT WAS FOUND THAT ARSENIC 6CH INCREASES THE EXPRESSION OF THIS PARTICULAR GENE SIGNIFICANTLY MORE THAN THE ANTIGEN. THUS ARSENIC ALBUM 6CH AS SUCH APPEARS DETRIMENTAL TO THE NORMAL HEALTH OF THE HUMAN BEINGS

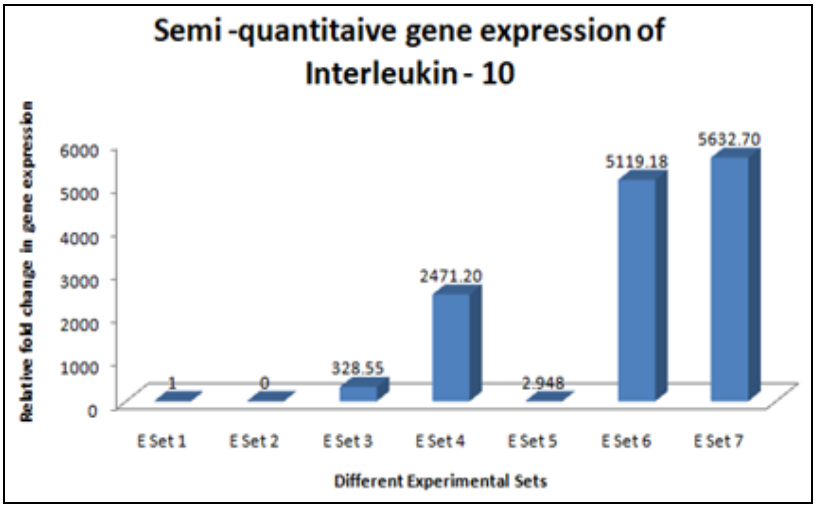


FIG. 5: IL -10 GENE EXPRESSIONS ARE DEFINITELY MUCH UP-REGULATED (MORE THAN 2000 TIMES) IF ARSENIC 6CH IS USED, THIS MAY COUNTERACT THE INCREASED IL- 6 GENE EXPRESSIONS BY THIS MEDICINE

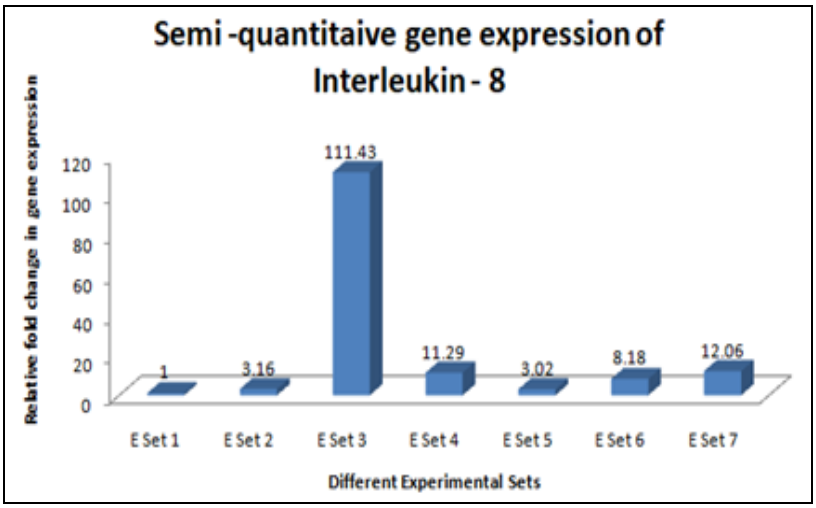


FIG. 6: ALTHOUGH ALCOHOL 6CH INCREASES IL- 8 GENE EXPRESSION MORE THAN 100 TIMES WHEN DIRECTLY USED, IT DECREASES IL-8 GENE EXPRESSION BY ABOUT 10 TIMES LESS. OTHER EXPRESSIONS ARE NOT SIGNIFICANT

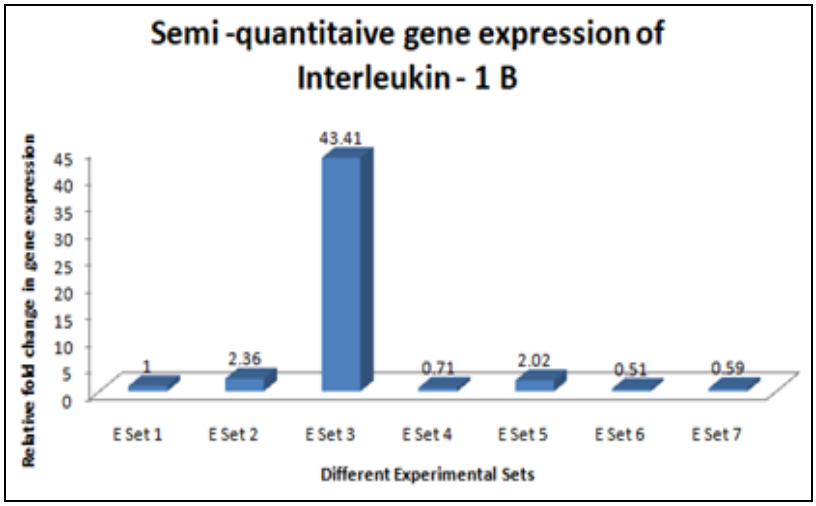


FIG. 7: ARSENIC 6CH MARKEDLY DECREASE IL-1 β GENE EXPRESSION INDUCED BY ALCOHOL 6CH. THUS WE MAY CONFER THAT IL-1 β IS ALSO MARKEDLY DECREASE BY ARSENIC 6CH

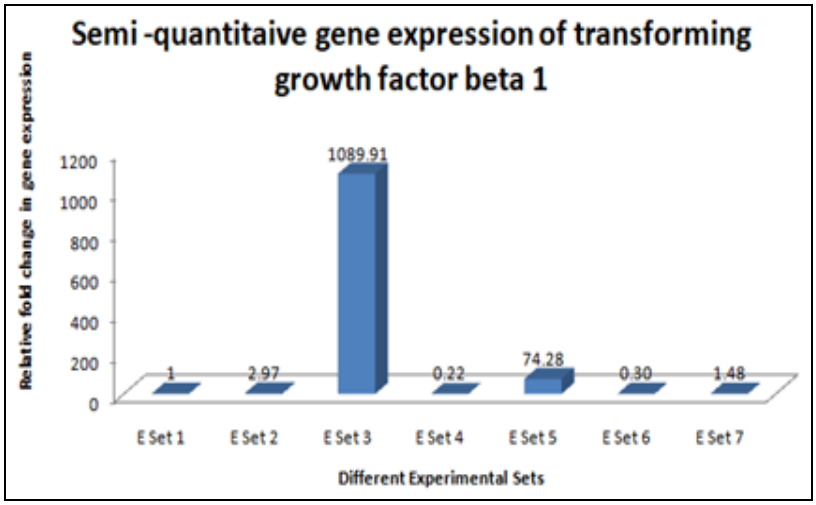


FIG. 8: TGF β1 IS MARKEDLY DECREASE BY ARSENIC 6CH

Where, E Set 1 – Experimental Set 1: Control (14th-day embryonated eggs).

E Set 2 - Alcohol control (14th-day embryonated eggs challenged with 70% v/v molecular grade ethanol).

E Set 3 - Potentized alcohol 6CH control (procured from HAPCO, embryonated eggs challenged with Alcohol 6CH).

E Set 4 - Medicine control (embryonated eggs challenged with Arsenic 6CH).

E Set 5 - Antigen control (embryonated eggs challenged with original spike protein (S) RBD antigen at 10µg/mL concentration dissolved in Phosphate buffer saline).

E Set 6 - To observe the pre-treatment action of the medicine against the S protein-induced pathological changes, ultra-diluted Arsenic album 6CH was inoculated first, followed by S antigen after one hour of incubation.

E Set 7 - To observe the post-treatment action of the medicine against the S protein-induced pathological changes, the S antigen was inoculated first, followed by ultra-diluted Arsenic album 6CH after one hour of incubation.

General Appearance of the Chick Embryo: The embryos were alive except for the embryos in which direct antigen was administered in all other experimental sets. The embryos of the direct antigen challenge group (antigen control) all embryos were dead and putrefied.

Among all the remaining experimental sets, when compared to the normal control antigen 6CH either directly or when used in preventive or curative sets

elicited better vitality and growth. The lungs were collapsed and inactivated. There were no significant macroscopic changes in the liver or intestines except in the direct antigen-challenged embryos, which showed gross hemorrhagic areas in different organs. Representative pictures of embryos in different sets of the experiment are depicted in **Fig. 9.**

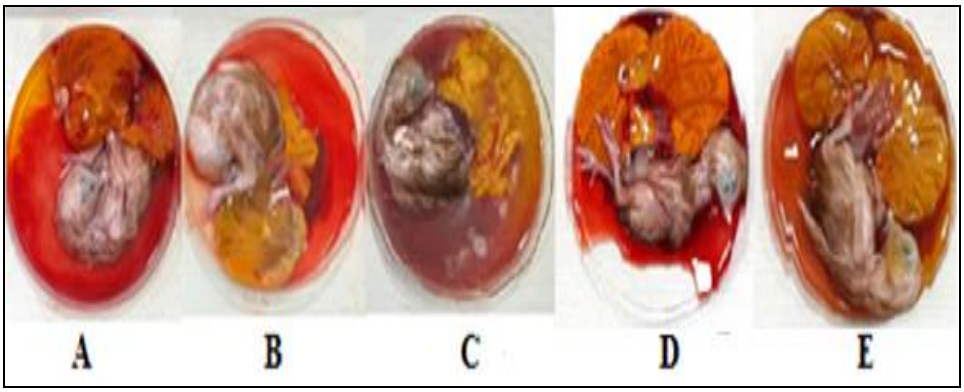


FIG. 9: SHOWING MACROSCOPIC APPEARANCE OF EMBRYOS IN EXPERIMENTAL SETS. A: BLANK (CONTROL); B: ALCOHOL 6C; C: DIRECT ANTIGEN; D: PRE ENTIVE SET (ARSENIC 6 C CHALLENGED BY ANTIGEN); E: CURATIVE SET (ANTIGEN CHALLENGED BY ARSENIC 6C).

Histological Changes:

Architecture: There were no architectural changes of the liver lobules except few pseudo lobular structures in the liver of direct antigen set embryos.

Hepatocytes: Few ballooning degenerations and Mallory-Denk bodies are present in hepatocytes where antigen was directly administered, while many Mallory-Denk bodies are present in hepatocytes in pre- treatment set experiment.

Other Changes: Lobular necroinflammation (acute hepatitis), apoptosis, massive bridging necrosis, interface hepatitis (Batts- Ludwig grade 3), portal mononuclear cell infiltration are present in an experimental set with the direct antigenic challenge. Mild necrosis, apoptosis, mild interface hepatitis (Batts- Ludwig grade 1) and few portal mononuclear cells are present in both pre and post-treatment experimental sets (Supplementary file1; Refer **Fig. 10-15.**

Representative Histological Pictures:

Liver:

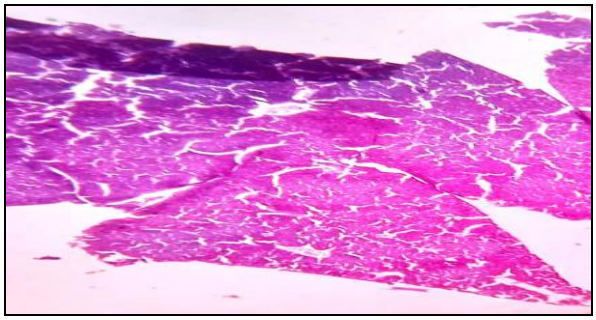


FIG. 10: CONTROL

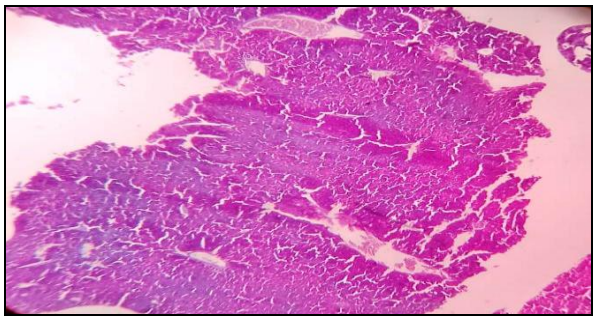


FIG. 11: ALCOHOL CONTROL

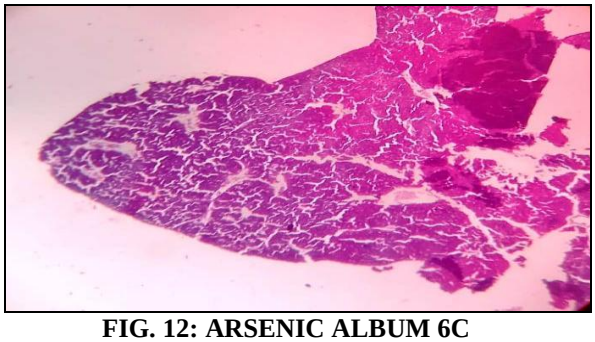


FIG. 12: ARSENIC ALBUM 6C

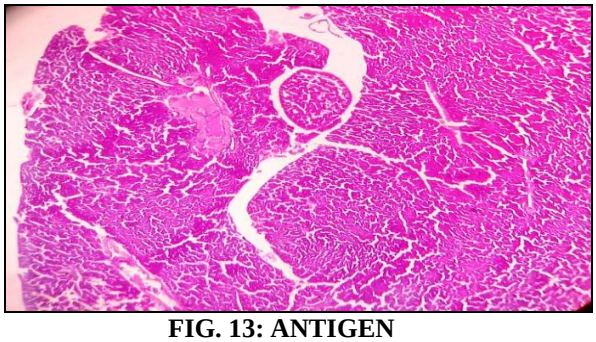


FIG. 13: ANTIGEN

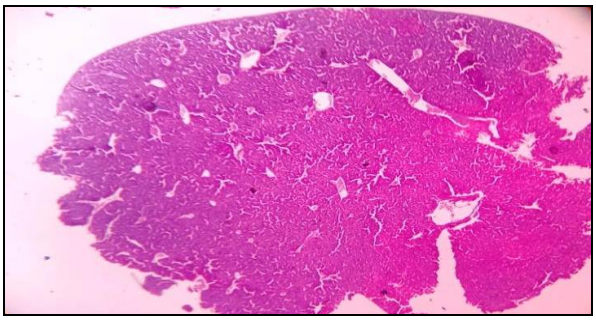


FIG. 14: ARSENIC ALBUM 6C CHALLENGED BY AG

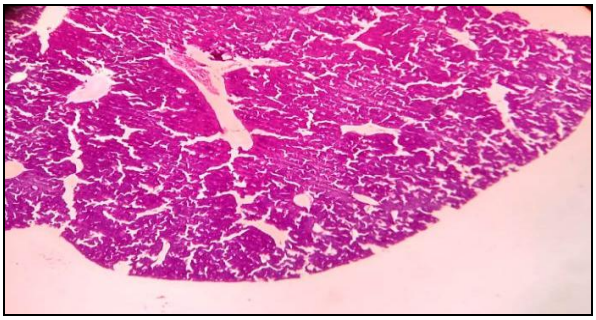


FIG. 14: AG CHALLENGED BY ARSENIC ALBUM 6C

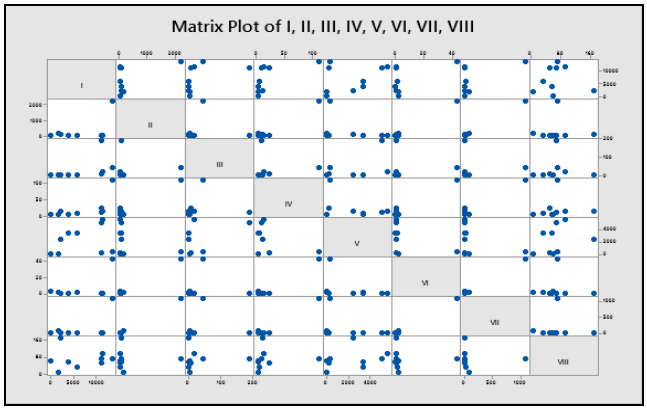
Data Analysis: ρ: pairwise Pearson correlation

TABLE 1: CORRELATIONS

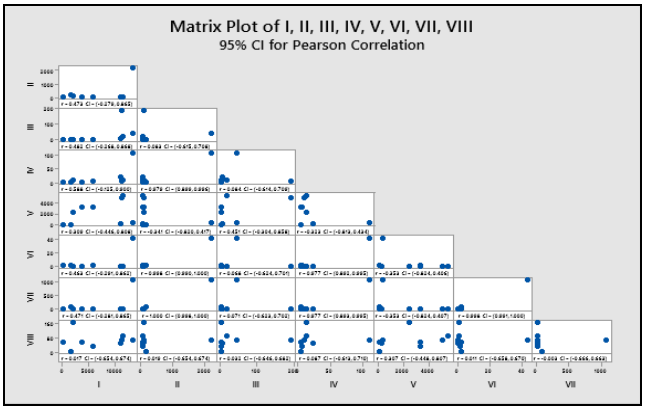
	I	II	III	IV	V	VI	VII
II	0.473						
III	0.482	0.083					
IV	0.588	0.979	0.084				
V	0.309	-0.341	0.451	-0.323			
VI	0.463	0.998	0.068	0.977	-0.353		
VII	0.471	1.000	0.071	0.977	-0.353	0.998	
VIII	0.017	0.019	0.032	0.087	0.307	0.011	-0.003

Where the numbers I – VIII denotes the following: I – Interferon-alpha (IFN α), II – Interferon-beta (IFN β), III – Interferon-gamma (IFN γ), IV – Interleukin 8 (IL -8). V – Interleukin 10 (IL -10), VI – Interleukin 1 beta (IL -1β), VII – Transforming growth factor beta 1 (TGF-β1), VIII – Interleukin 6 (IL -6).

Regression analysis and details of statistical analysis are given below in the **Table 2-16.**



MATRIX PLOT OF I, II, III, IV, V, VI, VII, VIII



CORRELATION: I, II, III, IV, V, VI, VII, VIII

Method

Correlation type	Pearson
Rows used	9

ρ: Pairwise Pearson Correlation

TABLE 2: PAIRWISE PEARSON CORRELATION

Sample 1	Sample 2	Correlation	95% CI for ρ	*P*-Value
II	I	0.473	(-0.279, 0.865)	0.199
III	I	0.482	(-0.268, 0.868)	0.189
IV	I	0.588	(-0.125, 0.900)	0.096
V	I	0.309	(-0.446, 0.808)	0.418
VI	I	0.463	(-0.291, 0.862)	0.210
VII	I	0.471	(-0.281, 0.865)	0.201
VIII	I	0.017	(-0.654, 0.674)	0.965
III	II	0.083	(-0.615, 0.708)	0.832

IV	II	0.979	(0.899, 0.996)	0.000
V	II	-0.341	(-0.820, 0.417)	0.369
VI	II	0.998	(0.990, 1.000)	0.000
VII	II	1.000	(0.998, 1.000)	0.000
VIII	II	0.019	(-0.654, 0.674)	0.962
IV	III	0.084	(-0.614, 0.709)	0.830
V	III	0.451	(-0.304, 0.858)	0.223
VI	III	0.068	(-0.624, 0.701)	0.862
VII	III	0.071	(-0.623, 0.702)	0.856
VIII	III	0.032	(-0.646, 0.682)	0.934
V	IV	-0.323	(-0.813, 0.434)	0.397
VI	IV	0.977	(0.892, 0.995)	0.000
VII	IV	0.977	(0.893, 0.995)	0.000
VIII	IV	0.087	(-0.613, 0.710)	0.825
VI	V	-0.353	(-0.824, 0.406)	0.351
VII	V	-0.353	(-0.824, 0.407)	0.352
VIII	V	0.307	(-0.448, 0.807)	0.421
VII	VI	0.998	(0.991, 1.000)	0.000
VIII	VI	0.011	(-0.658, 0.670)	0.977
VIII	VII	-0.003	(-0.666, 0.663)	0.995

Regression Analysis: I versus II, III, IV, V, VI, VII, VIII

Regression Equation:

I = 2751 - 44.8 II + 19.72 III + 499.1 IV + 1.167 V - 494 VI + 71.5 VII - 33.08 VIII

TABLE 3: COEFFICIENTS

Term	Coef	SE Coef	T-Value	*P-Value*	VIF
Constant	2751	583	4.72	*0.133*	
II	-44.8	19.8	-2.27	*0.264*	2935.79
III	19.72	5.79	3.40	*0.182*	1.85
IV	499.1	40.0	12.47	*0.051*	27.74
V	1.167	0.159	7.35	*0.086*	1.81
VI	-494	321	-1.54	*0.367*	295.34
VII	71.5	41.8	1.71	*0.337*	3242.28
VIII	-33.08	9.80	-3.38	*0.183*	2.86

Model Summary:

S	R-sq	R-sq(adj)	R-sq(pred)
748.921	99.73%	97.85%	0.00%

TABLE 4: ANALYSIS OF VARIANCE

Source	DF	Adj SS	Adj MS	F-Value	*P-Value*
Regression	7	208535984	29790855	53.11	*0.105*
II	1	2889498	2889498	5.15	*0.264*
III	1	6500997	6500997	11.59	*0.182*
IV	1	87250959	87250959	155.56	*0.051*
V	1	30305844	30305844	54.03	*0.086*
VI	1	1322404	1322404	2.36	*0.367*
VII	1	1637371	1637371	2.92	*0.337*
VIII	1	6392713	6392713	11.40	*0.183*
Error	1	560883	560883		
Total	8	209096866			

Regression Analysis: II versus I, III, IV, V, VI, VII, VIII

Regression Equation: II = 53.0 - 0.01868 I + 0.392 III + 9.29 IV + 0.0218 V - 9.15 VI + 1.662 VII - 0.562 VIII

TABLE 5: COEFFICIENTS

Term	Coef	SE Coef	T-Value	*P-Value*	VIF
Constant	53.0	22.0	2.41	*0.250*	
I	-0.01868	0.00823	-2.27	*0.264*	60.60
III	0.392	0.150	2.61	*0.233*	2.99
IV	9.29	4.25	2.19	*0.273*	752.05
V	0.0218	0.0103	2.12	*0.281*	18.09
VI	-9.15	7.80	-1.17	*0.450*	417.57
VII	1.662	0.308	5.39	*0.117*	422.25
VIII	-0.562	0.425	-1.32	*0.412*	12.89

Model Summary:

S	R-sq	R-sq(adj)	R-sq(pred)
15.2879	99.99%	99.96%	3.16%

TABLE 6: ANALYSIS OF VARIANCE

Source	DF	Adj SS	Adj MS	F-Value	*P-Value*
Regression	7	4220797	602971	2579.87	*0.015*
I	1	1204	1204	5.15	*0.264*
III	1	1587	1587	6.79	*0.233*
IV	1	1116	1116	4.78	*0.273*
V	1	1050	1050	4.49	*0.281*
VI	1	321	321	1.37	*0.450*
VII	1	6800	6800	29.09	*0.117*
VIII	1	409	409	1.75	*0.412*
Error	1	234	234		
Total	8	4221031			

Regression Analysis: III versus I, II, IV, V, VI, VII, VIII

Regression Equation:

III	=	-129.9 + 0.0467 I + 2.226 II - 23.23 IV - 0.0535 V + 23.0 VI - 3.60 VII + 1.483 VIII

TABLE 7: COEFFICIENTS

Term	Coef	SE Coef	T-Value	*P-Value*	VIF
Constant	-129.9	42.9	-3.03	*0.203*	
I	0.0467	0.0137	3.40	*0.182*	29.61
II	2.226	0.854	2.61	*0.233*	2318.48
IV	-23.23	7.34	-3.17	*0.195*	393.86
V	-0.0535	0.0207	-2.58	*0.236*	12.99
VI	23.0	17.1	1.35	*0.407*	352.65
VII	-3.60	1.80	-2.00	*0.295*	2534.22
VIII	1.483	0.787	1.89	*0.311*	7.78

Model Summary:

S	R-sq	R-sq(adj)	R-sq(pred)
36.4433	95.71%	65.69%	0.00%

TABLE 8: ANALYSIS OF VARIANCE

Source	DF	Adj SS	Adj MS	F-Value	*P-Value*
Regression	7	29639	4234	3.19	*0.407*
I	1	15394	15394	11.59	*0.182*
II	1	9017	9017	6.79	*0.233*
IV	1	13317	13317	10.03	*0.195*
V	1	8832	8832	6.65	*0.236*
VI	1	2407	2407	1.81	*0.407*
VII	1	5331	5331	4.01	*0.295*

VIII	1	4719	4719	3.55	*0.311*
Error	1	1328	1328		
Total	8	30967			

Regression Analysis: IV versus I, II, III, V, VI, VII, VIII

Regression Equation: IV = -5.45 + 0.001991 I + 0.0890 II - 0.0391 III - 0.002326 V + 0.984 VI - 0.1411 VII + 0.0664 VIII

TABLE 9: COEFFICIENTS

Term	Coef	SE Coef	T-Value	*P-Value*	VIF
Constant	-5.45	1.34	-4.07	*0.153*	
I	0.001991	0.000160	12.47	*0.051*	2.38
II	0.0890	0.0407	2.19	*0.273*	3127.17
III	-0.0391	0.0124	-3.17	*0.195*	2.11
V	-0.002326	0.000355	-6.55	*0.096*	2.26
VI	0.984	0.645	1.52	*0.370*	298.40
VII	-0.1411	0.0862	-1.64	*0.349*	3451.18
VIII	0.0664	0.0184	3.61	*0.172*	2.52

Model Summary:

S	R-sq	R-sq(adj)	R-sq (pred)
1.49573	99.98%	99.82%	0.00%

TABLE 10: Analysis of Variance:

Source	DF	Adj SS	Adj MS	F-Value	*P-Value*
Regression	7	9714.50	1387.79	620.32	*0.031*
I	1	348.02	348.02	155.56	*0.051*
II	1	10.68	10.68	4.78	*0.273*
III	1	22.43	22.43	10.03	*0.195*
V	1	95.96	95.96	42.89	*0.096*
VI	1	5.20	5.20	2.32	*0.370*
VII	1	6.00	6.00	2.68	*0.349*
VIII	1	29.18	29.18	13.04	*0.172*
Error	1	2.24	2.24		
Total	8	9716.74			

Regression Analysis: V versus I, II, III, IV, VI, VII, VIII

Regression Equation: V = -2290 + 0.841 I + 37.6 II - 16.26 III - 420.1 IV + 412 VI - 59.8 VII + 28.15 VIII

TABLE 11: COEFFICIENTS

Term	Coef	SE Coef	T-Value	*P-Value*	VIF
Constant	-2290	671	-3.41	*0.181*	
I	0.841	0.114	7.35	*0.086*	6.77
II	37.6	17.7	2.12	*0.281*	3287.75
III	-16.26	6.31	-2.58	*0.236*	3.05
IV	-420.1	64.1	-6.55	*0.096*	98.95
VI	412	284	1.45	*0.384*	319.07
VII	-59.8	36.9	-1.62	*0.352*	3512.95
VIII	28.15	8.06	3.49	*0.178*	2.69

Model Summary:

S	R-sq	R-sq(adj)	R-sq(pred)
635.678	98.99%	91.95%	0.00%

TABLE 12: ANALYSIS OF VARIANCE

Source	DF	Adj SS	Adj MS	F-Value	*P-Value*
Regression	7	39739463	5677066	14.05	*0.203*
I	1	21833782	21833782	54.03	*0.086*

II	1	1815624	1815624	4.49	*0.281*
III	1	2687184	2687184	6.65	*0.236*
IV	1	17332846	17332846	42.89	*0.096*
VI	1	851803	851803	2.11	*0.384*
VII	1	1057614	1057614	2.62	*0.352*
VIII	1	4928223	4928223	12.20	*0.178*
Error	1	404087	404087		
Total	8	40143550			

Regression Analysis: VI versus I, II, III, IV, V, VII, VIII

Regression Equation: VI = .02 - 0.001423 I - 0.0633 II + 0.0280 III + 0.711 IV + 0.00165 V + 0.1122 VII - 0.0458 VIII

TABLE 13: COEFFICIENTS

Term	Coef	SE Coef	T-Value	*P-Value*	VIF
Constant	4.02	2.58	1.56	*0.363*	
I	-0.001423	0.000926	-1.54	*0.367*	111.03
II	-0.0633	0.0540	-1.17	*0.450*	7604.77
III	0.0280	0.0208	1.35	*0.407*	8.29
IV	0.711	0.466	1.52	*0.370*	1306.94
V	0.00165	0.00113	1.45	*0.384*	31.96
VII	0.1122	0.0846	1.33	*0.411*	4606.02
VIII	-0.0458	0.0365	-1.25	*0.429*	13.78

Model Summary:

S	R-sq	R-sq(adj)	R-sq(pred)
1.27141	99.90%	99.19%	0.00%

TABLE 14: ANALYSIS OF VARIANCE

Source	DF	Adj SS	Adj MS	F-Value	*P-Value*
Regression	7	1601.39	228.770	141.52	*0.065*
I	1	3.81	3.811	2.36	*0.367*
II	1	2.22	2.222	1.37	*0.450*
III	1	2.93	2.929	1.81	*0.407*
IV	1	3.76	3.755	2.32	*0.370*
V	1	3.41	3.408	2.11	*0.384*
VII	1	2.84	2.843	1.76	*0.411*
VIII	1	2.54	2.540	1.57	*0.429*
Error	1	1.62	1.616		
Total	8	1603.01			

Regression Analysis: VII versus I, II, III, IV, V, VI, VIII

Regression Equation: VII = -30.1 + 0.01042 I + 0.582 II - 0.222 III - 5.16 IV - 0.01210 V + 5.68 VI + 0.301 VIII

TABLE 15: COEFFICIENTS

Term	Coef	SE Coef	T-Value	*P-Value*	VIF
Constant	-30.1	15.7	-1.91	*0.307*	
I	0.01042	0.00610	1.71	*0.337*	95.12
II	0.582	0.108	5.39	*0.117*	600.11
III	-0.222	0.111	-2.00	*0.295*	4.65
IV	-5.16	3.15	-1.64	*0.349*	1179.58
V	-0.01210	0.00748	-1.62	*0.352*	27.46
VI	5.68	4.28	1.33	*0.411*	359.45
VIII	0.301	0.288	1.04	*0.486*	16.95

Model Summary:

S	R-sq	R-sq(adj)	R-sq(pred)
9.04567	99.99%	99.94%	21.63%

TABLE 16: ANALYSIS OF VARIANCE

Source	DF	Adj SS	Adj MS	F-Value	*P-Value*
Regression	7	1039688	148527	1815.20	*0.018*
I	1	239	239	2.92	*0.337*
II	1	2381	2381	29.09	*0.117*
III	1	328	328	4.01	*0.295*
IV	1	219	219	2.68	*0.349*
V	1	214	214	2.62	*0.352*
VI	1	144	144	1.76	*0.411*
VIII	1	89	89	1.09	*0.486*
Error	1	82	82		
Total	8	1039770			

Regression Analysis: VIII versus I, II, III, IV, V, VI, VII

Regression Equation: VIII = 76.4 - 0.02779 I - 1.132 II + 0.526 III + 13.99 IV + 0.03283 V - 13.3 VI + 1.73 VII

TABLE 17: COEFFICIENTS

Term	Coef	SE Coef	T-Value	*P-Value*	VIF
Constant	76.4	28.3	2.70	*0.226*	
I	-0.02779	0.00823	-3.38	*0.183*	30.07
II	-1.132	0.856	-1.32	*0.412*	6570.25
III	0.526	0.279	1.89	*0.311*	5.12
IV	13.99	3.87	3.61	*0.172*	309.31
V	0.03283	0.00940	3.49	*0.178*	7.53
VI	-13.3	10.6	-1.25	*0.429*	385.67
VII	1.73	1.66	1.04	*0.486*	6078.95

Model Summary:

S	R-sq	R-sq(adj)	R-sq(pred)
21.7062	97.18%	77.43%	0.00%

TABLE 18: ANALYSIS OF VARIANCE

Source	DF	Adj SS	Adj MS	F-Value	*P-Value*
Regression	7	16225.7	2318.0	4.92	*0.334*
I	1	5370.1	5370.1	11.40	*0.183*
II	1	824.0	824.0	1.75	*0.412*
III	1	1674.2	1674.2	3.55	*0.311*
IV	1	6144.8	6144.8	13.04	*0.172*
V	1	5746.2	5746.2	12.20	*0.178*
VI	1	740.3	740.3	1.57	*0.429*
VII	1	513.7	513.7	1.09	*0.486*
Error	1	471.2	471.2		
Total	8	16696.8			

Where the numbers I – VIII denotes the following**:**

I – Interferon-alpha (IFN α).

II – Interferon-beta (IFN β).

III – Interferon gamma (IFN γ).

IV – Interleukin 8 (IL -8).

V – Interleukin 10 (IL -10).

VI – Interleukin 1 beta (IL -1β).

VII – Transforming growth factor-beta 1 (TGF-β1).

VIII – Interleukin 6 (IL -6).

DISCUSSION:

Interferons (IFN α, β, γ): Interferons (IFN) are considered to be the first line of antiviral response of the body against the infection [24]. The mechanisms of action of IFNs are they block the

replication of the virus within the body by several mechanisms. Finter *et al*. in 1991 have mentioned that though IFNs act in a slow manner against the acute stage of viral infections IFN alpha is considered to be an important player against chronic viral infection. Moreover, in addition to interferon-alpha, another important player of the innate antiviral response is interferon-gamma [24].

According to some scientific reports, several viral infections, such as herpes simplex virus (HSV), measles virus and vesicular stomatitis virus (VSV) which affect the central nervous system, the factors of JAK STAT pathway get activated, namely, Jaks 1 and 2 and STAT1 which in turn activates (interferon regulatory factor) IRF-1 and ultimately inhibits the protein synthesis of virus [25, 26]. The expressions of interferon-alpha were 11293.90 and 11533.06 in the respective pre-treatment, and post-treatment sets **Fig. 1**. The changes of interferon-alpha are probably by alcohol which is the vehicle of medicine Arsenic 6CH. But the expression of the interferon alpha gene is about five times less when only Arsenic 6CH is used. Thus, when it acts directly, arsenic may lower its influence on the IFN alpha gene expression.

However, in the pre-treatment and post-treatment experiments, this phenomenon was absent. This may be because in the presence of antigen, the Arsenic 6CH may lose its efficacy in lowering the level of interferon (IFN) alpha. Moreover, the changes of IFN beta gene expression are not so prominent. In most cases, Arsenic 6CH either directly or in pre-treatment and post-treatment roles decreased the significant expression of IFN beta gene expression, which was found with alcohol 6CH **Fig. 2.** In general, the Arsenic 6CH directly is not capable of increasing the interferon gene expressions and thus the increased interferon gene expressions with alcohol 6CH set appear to be complementary with Arsenic 6CH. In the case of IFN gamma, Arsenic 6CH decreases the expression of interferon-gamma than the controls. Still, if it is used as a preventive agent, it up-regulates the IFN gamma gene significantly, about 200 times more than the direct action of Arsenic 6CH. The curative treatment also up-regulates this gene more than 15 times **Fig. 3.** Thus, it is indeed a unique finding that although directly it does not change IFN gamma gene expressions but in COVID 19 conditions it may act as a good preventive and curative agent from the disease.

Interleukin 6 (IL- 6): As mentioned by the Sabaka *et al.* in 2021, there are several factors named as biomarkers or the inflammatory markers such as IL- 6, ferritin, C-reactive protein (CRP), fibrinogen, and D-dimer which are associated directly with the progression of the Covid – 19 infection [27]. Many other scientific reports have claimed the superiority or significance of IL-6 compared to the other biomarkers concerning the prediction of respiratory distress during the infection. IL-6 plays the most significant role in leading to immune dysregulation and ARDS during Covid -19 infection [28]. Moreover, it has also been noted that IL-6 is considered the robust marker of the condition hypoxemia, which suggests oxygen therapy for the patients. Patients who have been exposed to cytokine storm have revealed the initial rise of the levels of acute response cytokines, namely TNF and IL-1β and chemotactic cytokines - IL-8 and MCP-1, which created the condition of hypercytokinemia, which is in turn responsible for the persistent increase of the biomarker, IL-6 [28].

Thus, IL-6, in the process, binds with the IL-6 membrane receptor and stimulates the Janus kinase signal transducer, and induces the transcription of the JAK-STAT pathway leading to the inflammatory response. IL-6 also results in an elevation in the level of serum complement, CRP, ferritin, and pro-coagulant factors [29]. Our results have revealed that Arsenic album 6CH has a preventive role as the IL-6 level is 69.88 times in the Arsenic 6CH challenged by antigen set compared to 94.25 of the antigen challenged by arsenic 6CH set concerning housekeeping gene B-actin **Fig. 4.** Therefore, when we consider the IL-6 expression, it was found that Arsenic 6CH increases the expression of this particular gene significantly more than the antigen. Thus, Arsenic album 6CH appears detrimental to human beings' normal health.

Interleukin 10 (IL- 10): Another most important biomarker is the interleukin 10 (IL- 10), which is considered to be the master regulator of the inflammatory response. The critical role of the interleukin- 10 is to protect the tissues from damage induced by the severe phase of the immune

responses. Moreover, IL-10 can be secreted by all the immune system cells. The cytokine is considered to be a multifunctional one, and it has an impact upon the antiviral response [30].

In **Fig. 5** it can be noticed that there is a comparative secretion of the master immune regulator cytokine 5119.18 and 5632.70 fold more in the pre-treatment and post-treatment sets. IL- 10 gene expression is definitely much up-regulated (more than 2000 times) if Arsenic 6CH is used, this may counteract the increased IL- 6 gene expressions by this medicine.

Interleukin 8 (IL- 8): Although alcohol 6CH increases IL- 8 gene expression more than 100 times but when directly used it decreases IL-8 gene expression about 10 times less **Fig. 6**. Other expressions are not significant.

Interleukin 1 Beta (IL- 1β): Arsenic 6CH markedly decreases IL -1β gene expression induced by alcohol 6CH. Thus we may confer that IL- 1βis also markedly decreased by Arsenic 6CH **Fig. 7.**

Transforming Growth Factor (TGF β1): TGF β1 is markedly decreased by Arsenic 6CH **Fig. 8.**

CONCLUSION: Thus in this study, we elucidated predominant anti-inflammatory cytokine IL-10 gene expressions preventing pathological changes initiated by spike protein of the delta variant of SARS-CoV -2 in the fertilized chick embryo.

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SD planned the entire experiment, analyzed the data findings, and revised the final manuscript. PA facilitated in conceptualization and execution of the whole work.

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