Original Experimental Research 实验论著

Analysis of the capability of ultra-highly diluted glucose to increase glucose uptake in arsenite-stressed bacteria *Escherichia coli*

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Objective: Whether ultra-highly diluted homeopathic remedies can affect living systems is questionable. Therefore, this study sees value in the analysis of whether homeopathically diluted glucose 30C has any effect on *Escherichia coli* exposed to arsenite stress.
Methods: *E. coli* were cultured to their log phase in standard Luria-Bertani medium and then treated with either 1 mmol/L or 2 mmol/L sodium arsenite, with or without supplementation of either 1% or 3% glucose, an ultra-highly diluted and agitated ethanolic solution (70%) of glucose (diluted 10^{60} times), glucose 30C or 70% ethanol (placebo) in the medium. Glucose uptake, specific activities of hexokinase and glucokinase, membrane potential, intracellular adenosine triphosphate (ATP) and expression of glucose permease in *E. coli* were analyzed at two different time intervals. Arsenic content in *E. coli* (intracellular) and in the spent medium (extracellular) was also determined.
Results: In arsenite-exposed *E. coli*, the glucose uptake increased along with decreases in the specific activities of hexokinase and glucokinase, intracellular ATP and membrane potential and an increase in the gene expression level of glucose permease. Glucose uptake increased further by addition of 1%, 3% or ultra-highly diluted glucose in the medium, but not by the placebo.
Conclusion: The results demonstrated the efficacy of the ultra-highly diluted and agitated glucose in mimicking the action of actual glucose supplementation and its ability to modulate expressions of hexokinase and glucokinase enzymes and glucose permease genes, thereby validating the efficacy of ultra-high dilutions used in homeopathy.

Keywords: arsenites; glucose uptake; *Escherichia coli*; hexokinase; glucokinase; adenosine triphosphate; membrane potentials; homeopathy

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Homeopathy is an alternative method of treatment that often uses ultra-highly diluted medicines. The homeopathic method of dilution (potentiation) involves agitations (jerks or succussions) of the original drug in the ethanol vehicle (generally 40% to 90% ethanol) in each step of dilution. In the centesimal scale, 1 mL of the original drug (mother tincture) is mixed with 99 mL of ethanol and given 10 jerks to achieve a potency of 1C of the drug; again 99 mL of ethanol is mixed with 1 mL of the potency 1C and given 10 jerks to achieve a potency of 2C, and so on. Therefore, when the drug attains potency 12C and above, it becomes diluted to 10^{-24} (beyond Avogadro's limit) or above and the existence of even a single molecule of the original drug substance in such high potencies becomes highly improbable. Herein lays the controversy as to whether dilutions beyond Avogadro's limit can have any effect on living systems.

Although homeopathic clinicians and practitioners strongly believe that these diluted drugs can act to remove disease symptoms and cure patients of their conditions, others do not accept that any ultra-highly diluted drug can have demonstrable effects on any living system and question the scientific founding for the efficacy of such diluted drugs in ameliorating or curing any disease[1-5]. Furthermore, in some dubious cases, the reported effects could possibly be due to the so-called "placebo effects".

Numerous early attempts have been made to prove the efficacy of ultra-highly diluted homeopathic remedies, but most have not been accepted due to some lacunae in experimental design or for methodological shortcomings, or for some other flaws in the reports[3-5]. However, Khuda-Bukhsh *et al*[6-9] proposed a gene-regulatory hypothesis to explain the possible molecular mechanism of action of potentized homeopathic drugs with many circumstantial evidence to support their claim; this also needs to be further validated.

The present experiment was conducted to test primarily the hypothesis that a homeopathically potentized glucose diluted 10^{60} times (henceforth referred to as glucose 30C), far above the Avogadro's limit, could modulate metabolic activities in one of the prokaryotic organisms, *Escherichia coli*, under toxic stress. Furthermore, attempts were also made to understand the possible mechanisms involved. *E. coli*, commensal of the gut of warm-blooded animals and humans, can cause severe extra-intestinal diseases, such as septicemia and urinary tract infections[10]. Therefore, *E. coli* could be a suitable model prokaryotic organism with a simple genetic system for testing if an ultra-highly diluted homeopathically potentized solution of glucose (glucose 30C) could produce demonstrable effects in relation to glucose metabolism in this bacteria under some kind of physical stress such as simulating toxicity or disease stress.

Arsenic is a toxic metalloid widely disseminated in the environment and causes a variety of health and environment hazards. Due to the widespread occurrence of arsenic in the environment, every organism, from *E. coli* to human, has mechanisms for arsenic detoxification[11]. In bacteria, the genes for arsenic detoxification are usually encoded by arsenic resistance (ars) operons[12, 13]. Ars operon has three genes, arsR, arsB and arsC, where arsR is an arsenic (Ⅲ)-responsive transcriptional repressor[14]. The arsenic pump localized in the inner membrane of *E. coli* is composed of two types of subunits, the 45-kD ArsB protein[15, 16] and the 63-kD ArsA protein[17]. Dey *et al*[18] demonstrated that the ArsAB adenosine triphosphatase (ATPase) complex is a functional arsenite pump *in vitro*.

During glycolysis, arsenic inhibits the function of phosphoglyceral kinase in the glycolysis pathway. Therefore, on arsenic exposure, the glycolytic pathway remains incompletely utilized for the ultimate formation of adenosine triphosphate (ATP),

producing 4 mol instead of 8 mol of ATP (from 1 mol of glucose) produced by the normal pathway[19]. As the arsenic pump of the bacteria can only extrude arsenic at the expense of energy, by breakdown of ATP to adenosine diphosphate (ADP), and because on exposure to arsenic the ATP synthesis through glycolytic pathway is reduced to half, the first line of activity that would be expected in *E. coli* would be to increase glucose uptake (the ready source of energy) from the growth medium.

Hence, this study made glucose uptake an important parameter for the study and tested the efficacy of homeopathically prepared ultra-highly diluted glucose 30C (serially diluted 10^{60} times with agitations) on *E. coli* exposed to arsenite stress. The selection of glucose 30C as a possible remedy gains further significance because it is based on one of the homeopathic doctrines, namely, "like cures like", that is, glucose 30C could possibly have ameliorative effects on glucose metabolism-related toxicity or stress.

1 Materials and methods

1.1 Culture and treatment groups of *E. coli* *E. coli* were cultured to their log phase in the standard Luria-Bertani (LB) medium and were allocated randomly to the following sets: (1) standard LB medium — normal control; (2) LB supplemented with 1% and 3% glucose, respectively — glucose-supplemented control; (3) LB plus glucose 30C (in 70% alcohol) — glucose 30C-supplemented control; (4) LB plus 1 mmol/L sodium arsenite (Ⅲ) (Fluka, Switzerland) — arsenite-treated 1; (5) LB plus 2 mmol/L sodium arsenite — arsenite treated-2; (6) LB plus 1 mmol/L arsenite plus glucose 30C — arsenite-treated glucose 30C-supplemented 1; (7) LB plus 2 mmol/L arsenite plus glucose 30C — arsenite treated glucose 30C-supplemented 2; (8) LB plus 1 mmol/L arsenite plus alcohol 30C — arsenite-treated placebo-administered positive control 1; (9) LB plus 2 mmol/L arsenite plus alcohol 30C — arsenite-treated placebo-administered positive control 2.

From the growth curve (Figure 1), 1 mmol/L and 2 mmol/L sodium arsenite were chosen as sub-lethal doses in the experiments. From the control curve (where no arsenite was added) it was clear that the organism retained its log phase up to 1.5 h (90 min); hence all the experiments were performed at 45 min and 90 min.

The different parameters of study mentioned below have been conducted on three sub-sets of experiments each and for each sub-set of experiment, three replicates were studied ($n=9$). For normalization of the data against a standard growth pattern of the bacteria, the mean values were produced.

1.2 Selection of the dose of sodium arsenite *E. coli* were cultured to their log phase and then different doses of sodium arsenite (0 to 3.5 mmol/L) were administered in their growing medium and the optical density was measured at 600 nm wavelength under different time intervals.

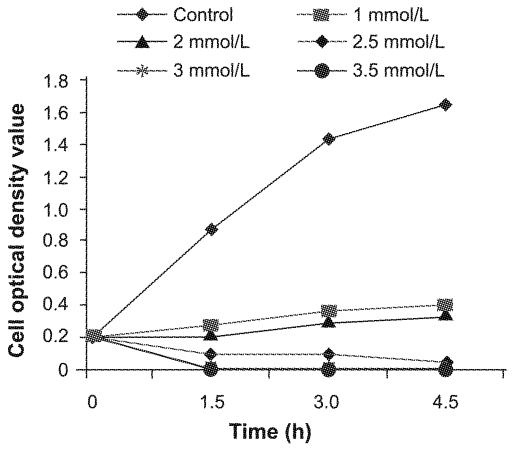

Figure 1 *E. coli* growth curve under different doses of sodium arsenite

1.3 Glucose uptake measurement Glucose uptake in *E. coli* of different sets was quantified by Anthron's method[20] (spectrophotometric analysis by Shimadzu PharmaSpec UV-1700) at 45 min and 90 min, respectively.

1.4 Hexokinase assay The specific activity of hexokinase[21] in *E. coli* of different sets was quantified by spectrophotometric study at 45 min and 90 min, respectively.

1.5 Glucokinase assay The specific activity of glucokinase[22] in *E. coli* of different sets was quantified by spectrophotometric study at 45 min and 90 min, respectively.

1.6 Total ATP measurement Total ATP contents in *E. coli* of different sets were measured by luminometric study[23, 24] at 45 min and 90 min, respectively, by using Thermo-Scientific Varioskan by EnzyLight ATP Assay Kit.

1.7 Measurement of membrane potential by spectrofluorimetric method Membrane potential of *E. coli* was determined spectrofluorimetrically by measuring the fluorescence quenching in intact cells[25] using the fluorescence dye 3,3′-diphenylthiocarbocyanine iodide. The incorporation of the dye into the intact cell membrane was known to be potential-dependent[26]. Relative membrane potential was calculated at 45 min and 90 min after the addition of arsenite by using the following equation: $(1-(b-a)/(c-a))\times 100\%$ (a = fluorescence intensity, b = fluorescence intensity with (cells+dye); c = fluorescence intensity with (cells + dye + carbonyl cyanide m-chlorophenylhydrazone (CCCP)).

1.8 Measurement of membrane potential by flow-cytometry method The change of membrane potential of the experimental cells was also analysed qualitatively by a flow-cytometer (FACS Calibur, Becton Dickson)[27]. Cells were cultured to their

log phase and the membrane potential was measured by a flow-cytometer using the same dye (fluorescence dye 3,3'-diphenylthiocarbocyanine iodide) after the addition of 1 mmol/L or 2 mmol/L sodium arsenite.

1.9 RNA isolation, cDNA preparation and gene level expression study by reverse transcription-polymerase chain reaction Total RNA was isolated from *E. coli* cells using TRIzol reagent (Bangalore Genei, India)[28]. Polymerase chain reaction (PCR) was performed using Taq DNA polymerase (Chromous Biotech, India) in accordance with the manufacturer's instruction. The primer sequences of amplified genes are shown in Table 1. PCR was performed on an automated thermal cycler (Applied Biosystems, USA). Amplified cDNA products were separated on 1.0% agarose gel electrophoresis in tris-acetic acid-ethylene diamine tetraacetic acid (TAE) buffer with 0.5 μg/mL ethidium bromide, observed under an ultraviolet trans-illuminator and photographed. Densitometry was performed on a negative image using the TotalLab software.

Table 1 Primer sequences used for this study

Primer name	Primer sequences (5′-3′)
G-3-PDH (house-keeping gene)	Forward: CCCACTAACATCAAATGGGG
	Reverse: CCTTCCACAATGCAAAGTT
ptsG (glucose permease)	Forward: CGGCGCTGACCTGGTTCCTG
	Reverse: ACGGAACCGCCTGCTTCTGC

1.10 Quantitative real-time reverse transcription-PCR Total RNA was isolated from *E. coli* cells using TRIzol reagent (Bangalore Genei, India), and after preparing cDNA a quantitative PCR was performed with SYBR Green Master Mix using an ABI7500 (Applied Biosystems, Inc., Foster City, CA, USA)[29]. Specific primers for glucose permease (ptsG F-5′-ACGCTTTGTGCCGATCATTTCTGG-3′ and ptsG R-5′-AACGCAACTACCGGGTTCTG-GTAA-3′) and control house-keeping gene glyceraldehyde-3-phosphatedehydrogenase (G-3-PDH) (F-5′-GAGAACGGGAAGCTTGTCATC-3′ and R-5′-CATGACGAACATGGGGGCATC-3′) were used in this study. The relative gene expressions were normalized to G-3-PDH using the $2^{-\Delta\Delta CT}$ ($\Delta\Delta CT = \Delta CT$ sample $- \Delta CT$ untreated control).

1.11 Arsenic measurement in spent medium and *E. coli* cells Arsenic measurements[30] in spent media and in *E. coli* cells of different sets were quantified by an atomic absorption spectroscope (AAS, Perkin-Elmer AA200, USA).

1.12 Preparation and source of glucose 30C and placebo 30C The glucose 30C and placebo 30C (70% ethanol) were prepared by the procedure of homeopathic serial dilutions and agitations; 1 mL of 1% glucose dissolved in 70% ethyl alcohol (the initial drug substance) was diluted with 99 mL of 70% ethanol and the mixture was given 10 hand jerks to produce potency 1; 1 mL of potency 1 was again mixed with 99 mL of 70% ethanol and agitated similarly to produce potency 2 and so on. The glucose 30C and placebo 30C were procured from Boiron Laboratory, Lyon, France.

The observers were blinded during observation and scoring of the data. The different coded vials (containing the randomized populations of *E. coli*) of both the experimental (containing the glucose 30C) and control sets (the placebo of vehicle) were not known to the observers as to which one belonged to the treated or control group; they were only deciphered later to remove any bias in observation.

1.13 Statistical analysis Data are presented as mean±standard error of mean (SEM). Statistical analysis was performed by one-way analysis of variance and Dunnett-*t* test. $P<0.05$ was considered significant.

2 Results

2.1 Glucose uptake The glucose uptakes (μg/mL per cell) in different control and treatment groups are summarized in Table 2.

Table 2 Results of glucose uptake analysis
(Mean±SEM, μg/mL per cell)

Group	*n*	Glucose uptake	
		45 min	90 min
Normal control	9	4.17±0.70	0.53±0.04
Normal control+1% glucose	9	114.50±4.50*	27.00±5.00*
Normal control+3% glucose	9	115.00±3.50*	29.50±2.00*
Normal control+placebo 30C	9	1.74±0.60	0.29±0.08
Normal control+glucose 30C	9	4.46±0.40*	0.62±0.07*
1 mmol/L arsenite	9	7.56±0.90*	2.87±0.50*
1 mmol/L arsenite+placebo 30C	9	7.47±1.00	2.90±0.59
1 mmol/L arsenite+glucose 30C	9	8.20±1.00△	3.12±0.40△
2 mmol/L arsenite	9	8.21±0.90*	3.10±0.25*
2 mmol/L arsenite+placebo 30C	9	8.12±1.00	2.98±0.50
2 mmol/L arsenite+glucose 30C	9	8.80±1.20▲	3.45±0.50▲

* $P<0.05$, *vs* normal control; △ $P<0.05$, *vs* placebo-treated positive control (1 mmol/L arsenite); ▲ $P<0.05$, *vs* placebo-treated positive control (2 mmol/L arsenite). SEM: standard error of mean.

The glucose uptake by *E. coli* significantly increased both at 45 min ($P<0.05$) and 90 min ($P<0.05$) after addition of 1% or 3% glucose as compared to that of the normal control cells. However, there was an overall decrease in glucose uptake when the data were compared between the 45 min and 90 min groups. In 1 mmol/L sodium arsenite treatment group, there was a significant

increase in glucose uptake both at 45 min ($P<0.05$) and 90 min ($P<0.05$) as compared to that of the control group. Glucose uptake further increased ($P<0.05$) when glucose 30C was added (in comparison with placebo-treated positive control group), but not when placebo was added. Almost the same results were observed when *E. coli* was exposed to 2 mmol/L sodium arsenite.

2.2 Hexokinase assay The specific activity of hexokinase in *E. coli* significantly decreased both at 45 min ($P<0.05$) and 90 min ($P<0.05$) after the addition of 1 mmol/L or 2 mmol/L sodium arsenite (Table 3). Specific activity of hexokinase decreased by 0.003 μmol/(mg · min) in 2 mmol/L arsenite insult than that of 1 mmol/L arsenite.

Addition of glucose 30C to 1 mmol/L and 2 mmol/L sodium arsenite-treated cells showed a significant decrease in their specific activity after 45 min ($P<0.05$) and 90 min ($P<0.05$), respectively, as compared to only 1 mmol/L and 2 mmol/L sodium arsenite treatments. Addition of glucose 30C to the normal medium also produced a decrease in specific activity as compared to that of the control set both at 45 min and 90 min. The addition of placebo did not make any significant difference in the specific activity of hexokinase in *E. coli* either at 45 min or 90 min.

Table 3 Specific activity of hexokinase
(Mean±SEM, μmol/(mg·min))

Group	*n*	Activity of hexokinase	
		45 min	90 min
Normal control	9	0.058±0.006	0.045±0.004
Normal control+glucose 30C	9	0.054±0.004*	0.036±0.002*
1 mmol/L arsenite	9	0.023±0.003*	0.021±0.001*
1 mmol/L arsenite+placebo 30C	9	0.020±0.004	0.020±0.003
1 mmol/L arsenite+glucose 30C	9	0.017±0.003△	0.015±0.005△
2 mmol/L arsenite	9	0.020±0.004*	0.017±0.002*
2 mmol/L arsenite+placebo 30C	9	0.020±0.002	0.016±0.002
2 mmol/L arsenite+glucose 30C	9	0.013±0.002▲	0.013±0.003▲

* $P<0.05$, *vs* normal control; △ $P<0.05$, *vs* placebo-treated positive control (1 mmol/L arsenite); ▲ $P<0.05$, *vs* placebo-treated positive control (2 mmol/L arsenite). SEM: standard error of mean.

2.3 Glucokinase assay The specific activity of glucokinase in *E. coli* significantly decreased both at 45 min ($P<0.05$) and 90 min ($P<0.05$) after the addition of 1 mmol/L and 2 mmol/L sodium arsenite (Table 4). Specific activity of glucokinase decreased approximately 0.001 μmol/(mg · min) in 2 mmol/L arsenite insult than that of 1 mmol/L arsenite.

Addition of glucose 30C to 1 mmol/L and 2 mmol/L sodium arsenite-treated cells showed a significant decrease in their specific activity after 45 min ($P<0.05$) and 90 min ($P<0.05$), respectively, as compared to only 1 mmol/L and 2 mmol/L sodium arsenite treatments. Addition of glucose 30C to the normal medium also produced a decrease in the specific activity as compared to that of control set both at 45 min (0.008 μmol/(mg · min)) and 90 min (0.004 μmol/(mg · min)) interval; however, the placebo did not.

Table 4 Specific activity of glucokinase
(Mean±SEM, μmol/(mg·min))

Group	*n*	Activity of glucokinase	
		45 min	90 min
Normal control	9	0.068±0.005	0.042±0.005
Normal control+glucose 30C	9	0.060±0.002*	0.038±0.002*
1 mmol/L arsenite	9	0.009±0.001*	0.007±0.001*
1 mmol/L arsenite+placebo 30C	9	0.009±0.002	0.007±0.001
1 mmol/L arsenite+glucose 30C	9	0.008±0.002△	0.003±0.001△
2 mmol/L arsenite	9	0.009±0.002*	0.006±0.001*
2 mmol/L arsenite+placebo 30C	9	0.009±0.002	0.006±0.001
2 mmol/L arsenite+glucose 30C	9	0.007±0.001▲	0.003±0.002▲

* $P<0.05$, *vs* normal control; △ $P<0.05$, *vs* placebo-treated positive control (1 mmol/L arsenite); ▲ $P<0.05$, *vs* placebo-treated positive control (2 mmol/L arsenite). SEM: standard error of mean.

2.4 Total ATP measurement in *E. coli* The data of total ATP concentrations in *E. coli* cells significantly decreased both at 45 min ($P<0.05$) and 90 min ($P<0.05$) after addition of 1 mmol/L and 2 mmol/L sodium arsenite treatment (Table 5).

Addition of glucose 30C to 1 mmol/L and 2 mmol/L sodium arsenite-treated cells showed a significant increase in ATP concentration both at 45 min ($P<0.05$) and 90 min ($P<0.05$), respectively, as compared to only 1 mmol/L and 2 mmol/L sodium arsenite treatments. Addition of glucose 30C to the normal medium also produced an increase in ATP concentration as compared to that of the control set both at 45 min (0.057 μmol/L per cell) and 90 min (0.065 μmol/L per cell) intervals. Addition of the placebo did not make any significant difference in ATP concentration either at 45 min or 90 min intervals. The overall concentration of ATP was found to be reduced at 90 min as compared to 45 min indicating that the ATP was degraded or utilized by the cell itself.

2.5 Assay of membrane potential of *E. coli* cells by spectroflurimetric study The membrane potential in *E. coli* significantly decreased both at 45 min ($P<0.05$) and 90 min ($P<0.05$) after addition of 1 mmol/L and 2 mmol/L sodium arsenite (Table 6).

Table 5 Results of intracellular ATP measurement
(Mean±SEM, μmol/L per cell)

Group	*n*	ATP measurement	
		45 min	90 min
Normal control	9	4.750±0.113	1.930±0.111
Normal control+glucose 30C	9	4.807±0.118*	1.995±0.115*
1 mmol/L arsenite	9	0.834±0.111*	0.579±0.115*
1 mmol/L arsenite+placebo 30C	9	0.834±0.115	0.575±0.113
1 mmol/L arsenite+glucose 30C	9	1.202±0.115△	0.779±0.112△
2 mmol/L arsenite	9	0.819±0.115*	0.559±0.113*
2 mmol/L arsenite+placebo 30C	9	0.817±0.118	0.584±0.119
2 mmol/L arsenite+glucose 30C	9	1.776±0.115▲	0.834±0.111▲

* $P<0.05$, *vs* normal control; △ $P<0.05$, *vs* placebo-treated positive control (1 mmol/L arsenite); ▲ $P<0.05$, *vs* placebo-treated positive control (2 mmol/L arsenite). ATP: adenosine triphosphate; SEM: standard error of mean.

Addition of glucose 30C to 1 mmol/L and 2 mmol/L sodium arsenite-treated cells showed a significant decrease in their membrane potential after 45 min ($P<0.05$) and 90 min ($P<0.05$) respectively as compared to only 1 mmol/L and 2 mmol/L sodium arsenite treatments. Addition of glucose 30C in normal medium also produced a decrease in membrane potential as compared to that of the control set both at 45 min (14.3 mV) and 90 min (5.4 mV) intervals. Placebo did not make any significant difference in membrane potential, measured in *E. coli* either at 45 min or at 90 min. Decreasing of membrane potential increased the amount of glucose uptake, that is, less membrane potential of cells indicated more glucose uptake.

Table 6 Results of cell membrane potential
(Mean±SEM, mV)

Group	*n*	Membrane potential	
		45 min	90 min
Normal control	9	68.80±2.00	94.10±2.65
Normal control+glucose 30C	9	54.50±1.45*	88.70±2.40*
1 mmol/L arsenite	9	33.50±1.25*	65.80±1.55*
1 mmol/L arsenite+placebo 30C	9	31.90±1.50	63.70±1.80
1 mmol/L arsenite+glucose 30C	9	15.80±1.60△	48.80±1.69△
2 mmol/L arsenite	9	30.20±1.50*	62.70±1.90*
2 mmol/L arsenite+placebo 30C	9	28.70±1.95	61.10±2.00
2 mmol/L arsenite+glucose 30C	9	10.30±1.60▲	44.70±1.60▲

* $P<0.05$, *vs* normal control; △ $P<0.05$, *vs* placebo-treated positive control (1 mmol/L arsenite); ▲ $P<0.05$, *vs* placebo-treated positive control (2 mmol/L arsenite). SEM: standard error of mean.

2.6 Assay of membrane potential of *E. coli* cells by flow-cytometry study When the flow-cytometry study was performed separately on the stained (with the fluorescence dye 3, 3′-diphenylthiocarbocyanine iodide) and unstained *E. coli* cells, maximum number of unstained cells were found to be located at the lower left region (Figure 2A), while the stained cells were maximally populated at the lower right region (Figure 2B). On the other hand, after treatment of the stained cells with 1 mmol/L and 2 mmol/L sodium arsenite, the location of the fluorescence was shifted towards the left (Figures 2C and 2D); this implied considerable release of fluorophores from the cells, which again signified the lowering of the membrane potential of the arsenite-treated cells. Again some intense fluorescence shifting towards the left side was observed when glucose 30C was added along with 1 mmol/L and 2 mmol/L sodium arsenite (Figures 2G and 2H).

2.7 Gene expression analysis by reverse transcriptase-PCR method The expression levels of glucose permease (GeneBank accession No. ECK1087) and house-keeping gene (G-3-PDH, GeneBank accession No. U82259) of *E. coli* cells in different control and treatment groups are summarized in Figure 3 and data are summarized in Table 7.

The arbitrary band intensity level significantly increased both at 45 min ($P<0.05$) and 90 min ($P<0.05$) intervals after the addition of 1 mmol/L and 2 mmol/L sodium arsenite. The arbitary band intensity level increased about 0.11 fold in 2 mmol/L arsenite insult than that of 1 mmol/L arsenite. Again the intensity of the band was known to be expression-dependent, that is, more intense band indicates more genetic level expression.

Addition of glucose 30C to 1 mmol/L and 2 mmol/L sodium arsenite-treated cells showed a significant increase in their band intensity after 45 min ($P<0.05$) and 90 min ($P<0.05$) respectively as compared to only 1 mmol/L and 2 mmol/L sodium arsenite treatments. Addition of glucose 30C in normal medium also produced little increase in band intensity as compared to that of control set both at 45 min (0.53 fold) and 90 min (0.8 fold) intervals. Addition of the placebo did not produce any significant difference in band intensity in *E. coli* either at 45 min or 90 min.

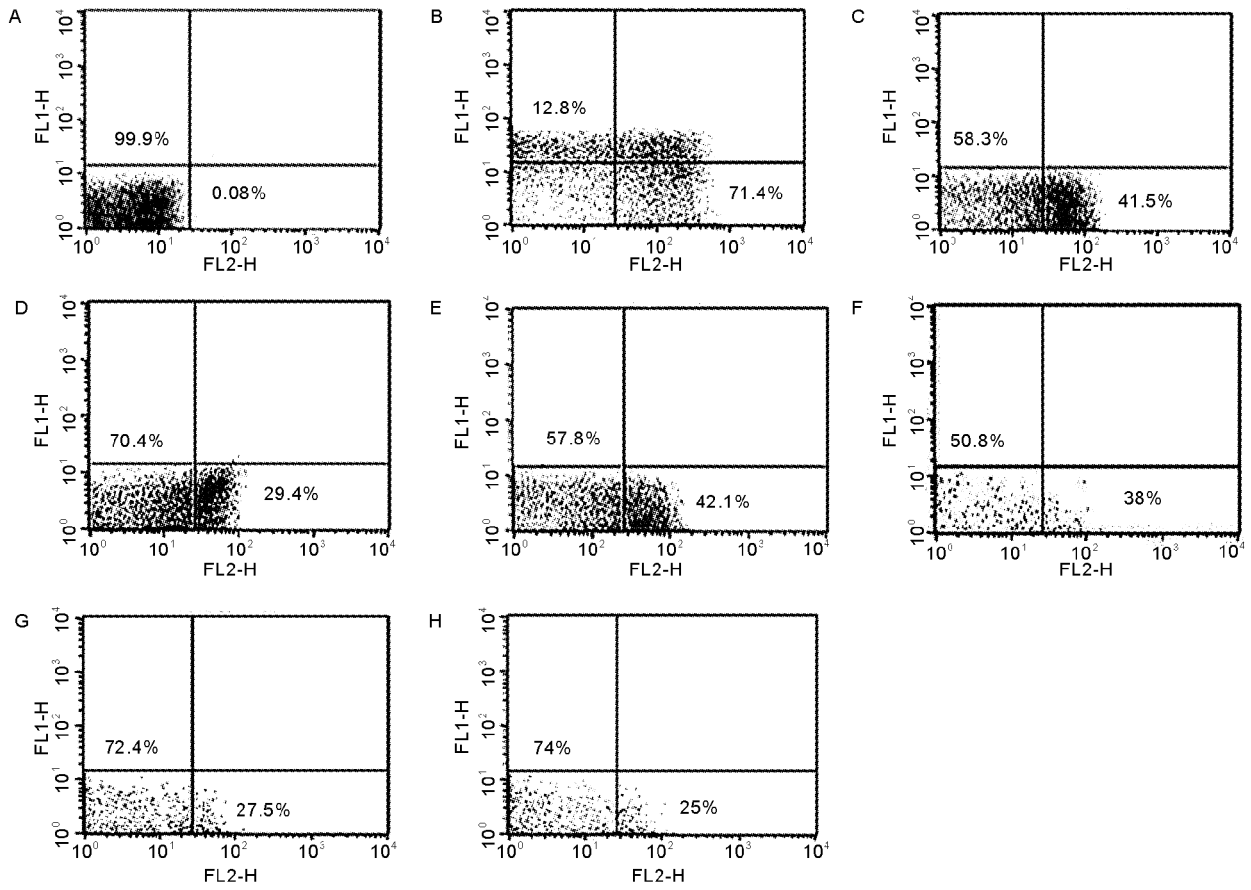

Figure 2 Analysis of membrane potential by flow-cytometry

Cell membrane potential was measured by a flow-cytometer after two different doses of sodium arsenite insult. A: Normal control cells; B: Glucose 30C-treated control cells; C: 1 mmol/L arsenite-treated cells; D: 2 mmol/L arsenite-treated cells; E: 1 mmol/L arsenite and placebo-treated cells; F: 2 mmol/L arsenite and placebo-treated cells; G: 1 mmol/L arsenite and glucose 30C-treated cells; H: 2 mmol/L arsenite and glucose 30C-treated cells.

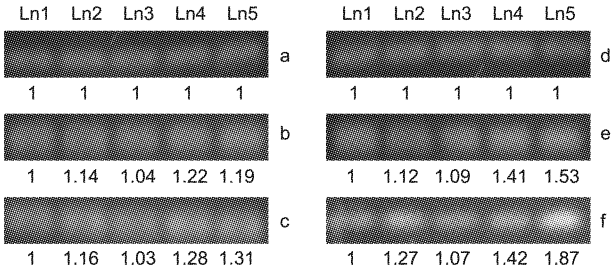

Figure 3 Gene expression analysis by reverse transcriptase-polymerase chain reaction

Arbitrary band intensities of ptsG (glucose permease gene) were analysed against the house keeping gene (G-3-PDH) under different conditions. (a) and (d) represent the expression of house keeping gene after 1 mmol/L and 2 mmol/L of sodium arsenite addition, respectively; (b) Expression of ptsG at 45 min after the addition of 1 mmol/L arsenite; (c) Expression of ptsG at 90 min after the addition of 1 mmol/L arsenite; (e) Expression of ptsG at 45 min after the addition of 2 mmol/L arsenite; (f) Expression of ptsG at 90 min after the addition of 2 mmol/L arsenite. The components of different lanes are: Ln1: Normal control cells; Ln2: Sodium arsenite-treated cells; Ln3: Sodium arsenite plus placebo-treated cells; Ln4: Sodium arsenite plus glucose 30C-treated cells; Ln5: Normal control plus glucose 30C-treated cells.

Table 7 Arbitrary band intensity of ptsG gene measured by RT-PCR method

(Mean±SEM)

Group	*n*	Band intensity	
		45 min	90 min
Normal control	9	1.00±0.10	1.00±0.09
Normal control+glucose 30C	9	1.19±0.06*	1.31±0.09*
1 mmol/L arsenite	9	1.14±0.04*	1.16±0.07*
1 mmol/L arsenite+placebo 30C	9	1.04±0.08	1.03±0.06
1 mmol/L arsenite+glucose 30C	9	1.22±0.05△	1.28±0.09△
2 mmol/L arsenite	9	1.12±0.10*	1.27±0.12*
2 mmol/L arsenite+placebo 30C	9	1.09±0.20	1.07±0.12
2 mmol/L arsenite+glucose 30C	9	1.41±0.12▲	1.42±0.15▲

* $P<0.05$, *vs* normal control; △ $P<0.05$, *vs* placebo-treated positive control (1 mmol/L arsenite); ▲ $P<0.05$, *vs* placebo-treated positive control (2 mmol/L arsenite). RT-PCR: reverse transcription-polymerase chain reaction; SEM: standard error of mean.

2.8 Gene expression analysis by quantitative real-time reverse transcription-PCR The relative gene expressions of ptsG (normalized by house-keeping gene G-3-PDH) of *E. coli* cells in different control and treatment groups are summarized in Table 8.

The relative gene expression level significantly increased both at 45 min ($P<0.05$) and 90 min ($P<0.05$) intervals after the addition of 1 mmol/L and 2 mmol/L sodium arsenite. The expression level increased to approximately 2.16 fold in 2 mmmol/L arseite-treated cells than that of 1 mmol/L arsenite-treated ones. Addition of glucose 30C to 1 mmol/L and 2 mmol/L sodium arsenite-treated cells showed a significant increase in their relative genetic expression after 45 min ($P<0.05$) and 90 min ($P<0.05$), respectively, as compared to only 1 mmmol/L and 2 mmol/L sodium arsenite treatments. Addition of glucose 30C in normal medium also increased glucose permease gene expression (by 0.22 fold and 0.56 fold at 45 min and 90 min, respectively). All expressions were measured against control cells (taking the expression of control cells as unity). Addition of the placebo did not make any significant difference in gene expression in *E. coli* either at 45 min or 90 min.

Table 8 Gene expression of ptsG measured by real-time PCR method

(Mean±SEM)

Group	*n*	Gene expression	
		45 min	90 min
Normal control	9	1.00±0.15	1.00±0.18
Normal control+glucose 30C	9	1.15±0.25*	1.56±0.88*
1 mmol/L arsenite	9	1.67±0.49*	4.11±0.89*
1 mmol/L arsenite+placebo 30C	9	1.50±0.49	3.18±1.25
1 mmol/L arsenite +glucose 30C	9	2.53±0.50△	5.02±0.65△
2 mmol/L arsenite	9	3.83±0.20*	6.72±0.45*
2 mmol/L arsenite+placebo 30C	9	2.28±0.19	6.20±0.44
2 mmol/L arsenite+glucose 30C	9	4.75±0.50▲	7.84±0.68▲

* $P<0.05$, *vs* normal control; △$P<0.05$, *vs* placebo-treated positive control (1 mmol/L arsenite); ▲$P<0.05$, *vs* placebo-treated positive control (2 mmol/L arsenite). PCR: polymerase chain reaction; SEM: standard error of mean.

2.9 Arsenic measurement in spent medium (extracellular) The arsenic concentration in spent medium significantly increased both at 45 min ($P<0.05$) and 90 min ($P<0.05$) after the addition of 1 mmol/L and 2 mmol/L sodium arsenite, respectively, compared to that of the control set (Table 9). Arsenic concentration in the spent media of 2 mmol/L arsenite insult was higher than that of 1 mmol/L arsenite treatment. Addition of glucose 30C to 1 mmol/L and 2 mmol/L sodium arsenite-treated cells showed a significantly increased concentration of arsenic in spent medium both at 45 min ($P<0.05$) and 90 min ($P<0.05$), respectively, as compared to that of only 1 mmol/L and 2 mmol/L sodium arsenite treatments. Addition of glucose 30C in normal medium also produced an increase in arsenic concentration in spent medium as compared to that of the control set both at 45 min (22.23 μg/L) and 90 min (25.68 μg/L) intervals, where the placebo did not.

Table 9 Results of extracellular arsenic measurement

(Mean±SEM, μg/L)

Group	*n*	Arsenic measurement	
		45 min	90 min
Normal control	9	196.67±17.0	227.32±20.0
Normal control+glucose 30C	9	218.90±21.0*	253.00±25.0*
1 mmol/L arsenite	9	295.79±23.0*	321.24±26.0*
1 mmol/L arsenite+placebo 30C	9	289.93±22.0	314.71±28.0
1 mmol/L arsenite+glucose 30C	9	337.50±22.0△	356.80±27.5△
2 mmol/L arsenite	9	368.18±26.0*	419.05±32.0*
2 mmol/L arsenite+placebo 30C	9	364.92±25.0	413.83±30.5
2 mmol/L arsenite+glucose 30C	9	398.00±24.6▲	445.80±31.8▲

* $P<0.05$, *vs* normal control; △$P<0.05$, *vs* placebo-treated positive control (1 mmol/L arsenite); ▲$P<0.05$, *vs* placebo-treated positive control (2 mmol/L arsenite). SEM: standard error of mean.

2.10 Arsenic measurement in *E. coli* cells (intracellular) The arsenic concentration in *E. coli* cells significantly increased both at 45 min ($P<0.05$) and 90 min ($P<0.05$) after the addition of 1 mmol/L and 2 mmol/L sodium arsenite, respectively, compared to that of the control set (Table 10).

Table 10 Results of intracellular arsenic measurement

(Mean±SEM, μg/L per cell)

Group	n	Arsenic measurement	
		45 min	90 min
Normal control	9	1.54±0.12	0.35±0.02
Normal control+glucose 30C	9	1.32±0.15*	0.22±0.05*
1 mmol/L arsenite	9	19.24±1.50*	2.79±0.80*
1 mmol/L arsenite+placebo 30C	9	19.62±1.80	2.81±0.70
1 mmol/L arsenite+glucose 30C	9	16.90±1.80△	2.54±0.90△
2 mmol/L arsenite	9	22.04±2.34*	3.44±1.10*
2 mmol/L arsenite+placebo 30C	9	21.82±2.00	3.50±0.85
2 mmol/L arsenite+glucose 30C	9	20.40±2.12▲	2.80±1.20▲

* $P<0.05$, *vs* normal control; △$P<0.05$, *vs* placebo-treated positive control (1 mmol/L arsenite); ▲$P<0.05$, *vs* placebo-treated positive control (2 mmol/L arsenite). SEM: standard error of mean.

The intracellular arsenic concentration decreased significantly after the addition of glucose 30C to 1 mmol/L and 2 mmol/L sodium arsenite-treated cells both at 45 min ($P<0.05$) and 90 min ($P<0.05$), respectively, compared to only arsenic-treated cells. Addition of glucose 30C in normal medium also produced a little decrease in intracellular arsenic concentration as compared to that of the control set both at 45 min (0.22 μg/L per cell) and 90 min (0.13 μg/L per cell) intervals. The addition of the placebo did not produce any significant difference in intracellular arsenic concentration either at 45 min or 90 min. However, the intracellular arsenic concentration of 2 mmol/L arsenite-intoxicated cells was 2 fold higher than that of 1 mmol/L arsenite-treated cells.

3 Discussion

When *E. coli* was exposed to a low dose of sodium arsenite ($AsNaO_2$), glucose uptake increased. This is due to the activation of the ars operon to expel arsenic from the cell. There are two types of arsenic resistant membrane proteins (ArsA and ArsB) which are located in the ars operon system[18, 31]. When ArsA is co-expressed with ArsB, an ArsAB complex is formed which is obligatorily coupled to ATP[32]. In *E. coli*, arsenite is extruded from the cells by the ArsAB ATPase pump. Initially ATP is bound to ArsA/ArsB protein which is converted to ADP after extruding arsenite from the bacteria. Hence ATP is continuously needed to extrude arsenite, for which more glucose uptake becomes necessary for replenishing ATP. Again, the production of ATP becomes half (4 mol of ATP was produced instead of 8 mol from 1 mol of glucose breakdown through glycolysis pathway) in the presence of arsenite. So, glucose uptake increases significantly to compensate the impaired production of ATP from glycolytic pathway and to increase the rate of ATP production, which is required for the extrusion of arsenic via energy-dependent functioning of ars operon.

In the present study, therefore, the increased uptake of glucose by *E. coli* in arsenite-supplemented medium was in conformity with the need of the organism, and it was quite expected that addition of 1% or 3% glucose in the medium would promote further glucose uptake. This increment in the glucose uptake rate was further supported by the data of specific activity of hexokinase and glucokinase, study of expression level of glucose carrier protein, glucose permease, and the study of membrane potential of *E. coli* cells under different conditions and the measurement of total ATP concentration. The specific activities of hexokinase and glucokinase were observed to decrease due to an increase in the glucose uptake rate, this is because the activity of an enzyme is always constant and if substrate concentration (glucose in this study) increases then the amount of enzyme (required to metabolize substrate) also increases and thus specific activity decreases. That is why specific activity of hexokinase and glucokinase was found to be decreased in arsenite-intoxicated cells, as compared to the control cells (because arsenic-treated cells showed higher uptake rate than the control cells). A decrease in the specific activity of hexokinase and glucokinase would depict that these enzymes were actively involved in the breakdown of glucose in the glycolytic pathway, signifying further that the enhanced glucose intake was partly utilized to gear up ATP production. This also compensated to some extent the loss caused due to the impaired activity of the phosphoglyceral kinase[19]. However, the overall extent of glucose uptake was found to be reduced at 90 min indicating that the requirement for glucose uptake was reduced after a certain period of time and there could be a threshold level or a saturation point after which glucose uptake would decline.

The concentration of ATP in the arsenite-treated cells was found to be decreased against the controlled cells. One explanation for this could be that the constant utilization of ATP by arsenite-treated cells for the extrusion of arsenic from the cells requires expenditure of energy for their survival. This statement would find support from the fact that the amount of arsenic present in the additional glucose 30C-treated spent medium (extracellular arsenic concentration) increased but the arsenic concentration within the cells decreased with the lapse of time.

Thus, the results would confirm that the arsenic extrusion mechanism in *E. coli* is primarily energy-dependent and the greater uptake of glucose was the primary step to ensure rapid utilization of ATP at the expense of which arsenic was being extruded from the cell. Gradual depletion of intra-cellular arsenic in the cell and its release into the medium with the lapse of time would explain the observed result showing an increase in extracellular arsenic concentrations as compared to the placebo control.

However, why the addition of glucose 30C that did not contain any original molecule of glucose not only increased glucose uptake by *E. coli*, but also showed an apparent increase in ATP concentration, was more challenging to explain with our current understanding. However, one hypothesis to explain this could be that the ultra-highly diluted glucose 30C had something in it ("molecular imprints" of glucose?), which resembled the original molecule of glucose. For this, they could mimic the role played by the original glucose molecules in increasing glucose uptake by gene induction and thereby increasing ATP production. To verify this contention, the data on the membrane potential of the arsenite, arsenite plus glucose 30C and only glucose 30C

treated cells were analyzed. The addition of glucose 30C to 1 mmol/L and 2 mmol/L sodium arsenite-treated cells showed a significant decrease in their membrane potential as compared to only 1 mmol/L and 2 mmol/L sodium arsenite treatments. The data revealed that the membrane potentials decreased significantly in all the cases, implicating thereby the uptake of an external substance (glucose in this study) into the cell system[33]. Therefore, the results of the flow-cytometry study were in strong conformity with those of the spectrofluorimetric study.

The increased expression level of glucose permease tested by RT-PCR and quantitative real-time PCR in arsenite-treated cells, arsenite plus glucose 30C-treated cells and only glucose 30C-treated cells would also provide additional support to this contention and that the glucose carrier protein also had a significant role in glucose insertion into the cells. However, in the absence of any physical presence of glucose molecule in glucose 30C, how exactly the modulations observed could be possible while the placebo 30C, which did not contain any initial glucose molecule (contained only 70% succussed ethanol) could not evoke any such responses, is a matter that needs further scientific scrutiny. Incidentally, in ultra-high dilutions of homeopathic drugs, some distinctive physico-chemical features have already been reported by some other studies[34-40]. The leading current proposal for the mode of action of such "ultra-molecular dilutions" is that water (or aquatic ethanol) is capable of storing information relating to substances with which it has been in contact and subsequently can transmit this information to a pre-sensitized biological system. The process is believed to be mediated by structural modification of water (or aquatic ethanol), analogous to storage of information by magnetic media[41]. Such information is retained in physical, rather than chemical form. Thus the molecular architechture of water (or aquatic ethanol) has a key role to play in understanding homeopathic mechanisms of action[42, 43]. Therefore, further studies on their physico-chemical properties that make glucose 30C so different in action in regard to glucose uptake in *E. coli*, are warranted for understanding the mechanistic principle more precisely, which may reveal a gene regulatory role of ultra-highly diluted agitated solutions (Tables 7 and 8) or understanding the ultimate molecular mechanism of biological action, as proposed earlier by Khuda-Bukhsh[6-9]. Finally, it seems that the "memory of water" hypothesis proposed originally by Davenas *et al*[44] should again be put to more elaborate scientific explorations, preferably by well-designed multi-center studies.

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5 Competing interests

The authors declare that they have no competing interests.

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高度稀释的葡萄糖溶液提高含亚砷酸盐培养基中大肠埃希氏杆菌的葡萄糖摄取

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目的:高度稀释的顺势疗法药物对活体系统的作用一直被质疑。因此,本研究检测依据顺势医学理论而高度稀释的葡萄糖溶液对暴露于亚砷酸盐的大肠埃希氏杆菌的作用。

方法:大肠埃希氏杆菌在 Luria-Bertani 培养基中培养至对数期后分组。分别加入 1%或 3%的葡萄糖溶液、葡萄糖 30C(在 70%乙醇中稀释 10^{60} 倍)、1 mmol/L 或 2 mmol/L 的亚砷酸钠、1 mmol/L 或 2 mmol/L 的亚砷酸钠加葡萄糖 30C、1 mmol/L 或 2 mmol/L 的亚砷酸钠加乙醇 30C(安慰剂)。分析用药后 45 min 及 90 min 大肠埃希氏杆菌的葡萄糖摄取、己糖激酶及葡糖激酶活性、细胞膜电位、细胞内三磷酸腺苷含量以及葡萄糖通透酶基因的表达情况,并测定细胞内及细胞外(培养基内)亚砷酸盐的浓度。

结果:暴露于亚砷酸盐的大肠埃希氏杆菌的葡萄糖摄取量增加,己糖激酶及葡糖激酶活性、细胞内三磷酸腺苷含量及细胞膜电位降低,葡萄糖通透酶基因的表达增加。在加入 1%或 3%葡萄糖或高度稀释的葡萄糖 30C 的培养基内,大肠埃希氏杆菌的葡萄糖摄取量进一步增加,而在加入乙醇 30C(安慰剂)的培养基内,大肠埃希氏杆菌的葡萄糖摄取量没有明显增加。

结论:本研究的结果证实了高度稀释的葡萄糖溶液对于真正的葡萄糖溶液的模仿作用。这种顺势疗法理论下高度稀释的葡萄糖溶液能够调节大肠埃希氏杆菌己糖激酶和葡糖激酶的表达以及葡萄糖通透酶基因的表达,证实了顺势疗法中高度稀释的药物的有效性。

关键词:亚砷酸盐类;葡萄糖摄取;大肠杆菌;己糖激酶;葡糖激酶;腺苷三磷酸;膜电位;顺势疗法