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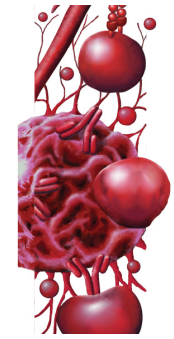

Double-blind, placebo-controlled, 1:1 randomized Phase III clinical trial of Immunoxel honey lozenges as an adjunct immunotherapy in 269 patients with pulmonary tuberculosis

Aim: Safer and shorter antituberculosis treatment (ATT) regimens represent the unmet medical need. **Patients & methods:** The patients were randomly assigned into two arms: the first (n = 137) received once-daily sublingual honey lozenge formulated with botanical immunomodulator Immunoxel and the second (n = 132) received placebo lozenges along with conventional ATT. Immunoxel and placebo arms were demographically similar: 102 versus 106 had drug-susceptible TB; 28 versus 20 multidrug-resistant TB (MDR-TB); 7 versus 7 extensively drug resistant TB (XDR-TB); and 22 versus 20 TB-HIV. The primary end point was sputum smear conversion. **Results:** After 1 month 87 out 132 (65.9%) of Immunoxel recipients became sputum smear negative, whereas 32 out of 127 (25.2%) in placebo group had converted ($p < 0.0001$). Sputum clearance produced by Immunoxel was equally effective across all forms of TB. In the immunotherapy arm the average weight gain was 2 kg, but placebo recipients gained only 0.6 kg. Immunoxel reduced TB-associated inflammation as evidenced by defervescence and normalization of elevated leukocyte counts and erythrocyte sedimentation rate. No adverse effects were seen at any time. The liver function tests indicate that ATT-caused hepatotoxicity was counteracted by Immunoxel. These results are in agreement with prior 20 trials of Immunoxel conducted over the past 17 years. **Conclusion:** Immunoxel is affordable, safe, effective, fast-acting, commercially available immunotherapeutic intervention to supplement conventional TB chemotherapy. (Clinicaltrials.gov ID NCT01061593).

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Drug-susceptible tuberculosis (DS-TB) is curable with the first line of antituberculosis treatment (ATT) in 85% of cases within 6 months. Nevertheless, despite the availability of effective treatment, the TB epidemic does not seem to go away [1]. Among many challenges posed by *Mycobacterium tuberculosis* infection are the difficult-to-treat forms of TB, such as MDR-TB, XDR-TB and TB with HIV. It takes as long as 24 months to treat a patient with MDR-TB and the typical success rate is less than 50% [2]. Treating XDR-TB for 2 years resulted in favorable outcome in only 16% of patients of whom 41% had HIV [3]. TB refractory to conventional ATT requires the deployment of second-line drugs, however the cost of such a therapy increases by orders of magnitude without substantial benefit of better safety or efficacy.

Eastern Europe and Central Asia have one of the largest TB prevalence rates globally, often in parallel with HIV epidemic. While the incidence of pulmonary TB in Ukraine had decreased from 77.8 in 2008 to 68.1 in 2012, the co-infection with HIV had increased from 8.7 in 2011 to 10.4 in 2012 [4].

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The incidence of MDR-TB was 15.3 per 100,000 in 2012 and there is a serious concern that it may increase further due to ongoing military conflict and resulting economic hurdles [5]. The favorable treatment outcome for drug-resistant TB in Ukraine was 55.7%. Mongolia has one of the highest TB burdens in the Western Pacific region and tuberculosis is among the top ten causes of mortality. In 2012 the incidence of TB was 139 per 100,000. The WHO estimates the current incidence to be as high as 230 per 100,000 [6]. In terms of HIV infection Mongolia is a low-prevalence country with 127 cases reported in 2012 versus 36 in 2007 [7]. The rate of MDR-TB in Mongolia is not well known; based on published evidence it appears to be somewhat lower than in Ukraine [8]. In a 2007 survey 1.4% of new TB cases and 28% of retreatment cases had MDR-TB [9]. While treatment outcomes in first-diagnosed TB cases were satisfactory, only 69% of relapsed TB patients were successfully treated, suggesting that it might have been due to drug resistance [9,10].

It is clear that better treatment options are urgently needed. If such an intervention is found, the impact on the healthcare and clinical management of TB will be substantial. Significant efforts are directed at finding new TB drugs [2]. Immune-based interventions are actively sought as an adjunct therapy to conventional ATT [11]. Immunoxel is an alcohol–water phytoconcentrate of medicinal plants approved by the Ministry of Health of Ukraine as a dietary supplement with immunomodulating properties. Immunoxel (Dzherelo) is the most popular botanical product of Ukrainian company Ekomed, which so far has been used by over 500,000 individuals for various indications including chronic bacterial and viral infections such as TB and HIV, autoimmune diseases and malignancy [12]. Seventeen years ago, Ukrainian TB doctors accidentally discovered that when Immunoxel was used along with ATT it cut down TB treatment time, increased bacillary clearance rate and lowered drug-induced hepatotoxicity [13]. The efficacy was the same regardless whether a patient had either DS-TB, or any of MDR-TB, XDR TB, or TB with HIV. Since then 20 clinical trials involving close to 1500 patients with TB and HIV were conducted by us. We have recently compared the efficacy of Immunoxel formulated into various solid dosage forms, which are more convenient to use than the liquid formula. Based on the preliminary Phase II study, the best results were obtained with Honibe honey lozenges manufactured in Canada by Island Abbey Foods Ltd [14]. The aim of the present study was to confirm the clinical benefit of Immunoxel honey lozenges versus placebo in a representative population of Ukrainian and Mongolian patients with DS-TB, MDR-TB, XDR-TB and TB-HIV.

Materials & methods

Clinical setting & baseline patients' characteristics

The study was conducted at three Mongolian and five Ukrainian TB hospitals and dispensaries between November 2013 and January 2015. The conduct of the study was approved by the internal review board of the lead hospital in respective countries in accordance with the Helsinki Declaration. The main eligibility criteria were an informed consent and positive sputum smear. Patients in each country were randomly allocated by computer-generated sequence into two groups to receive in double-blinded fashion either Immunoxel honey lozenges or identically appearing placebo lozenges. All patients had one or more TB symptoms such as low-grade fever, cough, chest pain, dyspnea, hemoptysis, weight loss and anorexia. The randomization resulted in equal distribution of baseline characteristics: ethnicity, age, gender, height, body weight, blood cell counts, serum biochemistry parameters, severity and various manifestations of the disease. Thus, the statistical bias due to population heterogeneity was unlikely (Table 1). Some of the patients required individualized treatment rather than standard ATT regimen with first-line TB drugs; the proportion of such patients was 35/137 (25.5%) and 26/132 (19.7%) in Immunoxel and placebo arms, respectively; the inter-group difference was not statistically significant ($p = 0.31$). Remaining patients were prescribed conventional 2HREZ/4HR regimen supplemented with streptomycin (S). The number of patients with DS-TB, MDR-TB, XDR-TB and TB-HIV was 102:28:7:22 and 106:19:7:20 in Immunoxel and placebo arms, respectively ($p = 0.99$). The distribution of HIV-positive individuals among DS-TB, MDR-TB and XDR-TB was 17:3:2 and 19:1:0 in Immunoxel and placebo arms, respectively. In Mongolia all of the patients, except one in each arm, were categorized as DS-TB since information on their drug resistance status and HIV infection was not readily available.

Immunoxel

Immunoxel is a water-alcohol extract of aloe (*Aloe arborescens*), licorice (*Glycyrrhiza glabra*), dog rose fruit (*Rosa canina*), oregano (*Oreganum majorana*), sage (*Salvia officinalis*), thyme (*Thymus vulgaris*), fennel (*Foeniculum vulgare*), dandelion (*Taraxacum officinale*), purple coneflower (*Echinacea purpurea*), nettle (*Urtica dioica*), marigold (*Calendula officinalis*), greater plantain (*Plantago major*), wormwood (*Artemisia sp.*), common knotgrass (*Polygonum aviculare*), yarrow (*Achillea millefolium*), centaury (*Centaurium erythraea*), elecampane (*Inula helenium*), tormentil (*Potentilla erecta*), cudweed (*Gnaphalium uliginosum*),

Table 1. Baseline characteristics of patients with pulmonary TB enrolled to receive either Immunoxel or placebo in combination with conventional TB drugs.

Characteristics	Immunoxel n = 137 (%)	Placebo n = 132 (%)	p-value
Asian	50 (36.5%)	50 (37.9%)	0.90
Caucasian	87 (63.5%)	82 (62.1%)	0.90
Females	44 (32.1%)	40 (30.3%)	0.79
Males	93 (67.9%)	92 (69.7%)	0.79
Age	37.2	38.4	0.40
Height cm	169.9	170.2	0.76
Weight kg	58.8	58.2	0.62
BMI kg/m^2	20.2	20.1	0.62
Axillary temperature (°C)	37.4	37.7	0.02
AFP-positive smears	132 (96.4)	127 (96.2)	1.0
Mean sputum score	1.31	1.33	0.87
DS-TB	102 (74.5%)	106 (80.3%)	0.31
MDR-TB	28 (20.4%)	19 (14.4%)	0.20
XDR-TB	7 (5.1%)	7 (5.3%)	1.0
TB-HIV	22 (16.1%)	20 (15.2%)	0.74
DS-TB-HIV	17 (12.4%)	19 (14.4%)	0.72
MDR-TB-HIV	3 (2.2%)	1 (0.8%)	0.62
XDR-TB-HIV	2 (1.5%)	0	0.5
Patients with cancer	0	0	1.0
Patients with diabetes	1	1	1.0
Leukocytes × 10^9 l	7.7 (20.9)	7.8 (20.9)	0.82
Lymphocytes (%)	23.5 (38.2)	23.1 (35.9)	0.78
Mean CD4 lymphocytes in TB-HIV subjects	309 ± 156	306 ± 228	0.96
Hemoglobin (g/l)	121.6 (46.7)	119.4 (52.3)	0.42
ESR (mm/h)	21.5 (45.4)	23.8 (47.3)	0.18
ALT (mM)	0.243	0.273	0.41
Total bilirubin (g/l)	12.6 (4.3%)	11.4 (2.4)	0.12

The cut-off values for following end points were: normal BMI > 18.5 kg/m^2.
Lymphocytes 20–40%; normal leukocyte counts 4–9 cells/10^{9}l; ESR < 20 mm/h.
Hemoglobin 120–175 g/l; ALT < 0.68 mM/h*l; total bilirubin 3–19 g/l.
ALT: Alanine transaminase; ESR: Erythrocyte sedimentation rate.

three-lobe beggarticks (*Bidens tripartita*), Siberian golden root (*Rhodiola rosea*), chaga (*Inonotus obliquus*), snowball tree berries (*Viburnum opulus*), seabuckthorn berries (*Hippophae rhamnoides*) and juniper berries (*Juniperus communis*) according to standardized manufacturing process using each plant at a specific proportion as approved by the Ministry of Health of Ukraine in 2006 (TUU 15.8–23732912–016:2006). Immunoxel was produced by Ukrainian company Ekomed in Kiev, Ukraine, shipped to Island Abbey Food Science company in Canada, and formulated into proprietary Honibe honey lozenges at 300 µl dose per lozenge. The preparation is stable at ambient temperature with a shelf life of 3 years.

Treatment regimen

The TB drugs were provided free-of-charge from the national distribution system administered by the respective Ministries of Health. Standard TB therapy consisted of daily doses of isoniazid (H) 300 mg; rifampicin (R) 600 mg; pyrazinamide (Z) 2000 mg; ethambutol (E) 1200 mg; and streptomycin (S) 1000 mg. Individualized therapy for drug-resistant patients comprised first- and second-line TB drugs as decided

empirically by a physician. The choice of second-line drugs was as follows: aminoglycosides such as kanamycin (K) and amikacin (A); thioamides for example, ethionamide (T); fluoroquinolones, for example, levofloxacin (L), sparfloxacin (F), and gatifloxacin (G); cycloserine (Cs); para-aminosalicylic acid (P); and clofazimine (C). In the category of individualized therapy were also patients who ended up with complete treatment failure and were receiving so-called 'palliative' regimen consisting of life-long H and R only. In the immunotherapy group, in addition to ATT, patients received once-daily sublingual lozenges of Immunoxel which was given 30 min before or after breakfast. In the control group patients received placebo lozenges made of corn syrup, with the same appearance and excipients, but without Immunoxel. All patients were enrolled at the beginning of their TB treatment. No other immunomodulators or anti-infective drugs besides ATT were used during this study.

Laboratory assays

A standard microscopy examination of sputum smear staining by Ziehl–Neelsen or acid-fast bacteria was conducted prior to study entry and at post-treatment time. Sputum smears were scored by blinded laboratory technician from 0 to 3; those who had bacterial burden less than 1 were assigned 0.1. Drug resistance profile to first- and second-line TB drugs was done in Ukraine only, by using commercially supplied kit (Tulip Diagnostics, Goa, India) with ready-to-use tubes containing TB drugs incorporated at manufacturer-predetermined concentrations into standard Löwenstein–Jensen agar slants. The MDR-TB was diagnosed when resistance to both isoniazid and rifampicin, with or without resistance to additional drugs, was present. When there was an additional resistance to any fluoroquinolones and amikacin, kanamycin or capreomycin, then it was categorized as XDR-TB. The samples of peripheral blood of patients with TB-HIV were analyzed by fluorescent microscopy using commercially available Clonospectr panel of monoclonal antibodies against surface CD3, CD4 and CD8 antigens of T lymphocytes (MedBioSpectr, Moscow, Russia). Other tests such as complete blood count and biochemistry analysis were carried out by routine methods.

Statistical analysis

Data collected were analyzed with statistical software available online (GraphPad Software Inc., CA, USA). All statistical analyses were done on intent-to-treat basis, involving the total number of patients. Where necessary the analysis of subgroups within responder and nonresponder populations was carried out. Parametric baseline values relative to the end of study results were evaluated by paired Student t-test and intergroup differences were evaluated by unpaired t-test. The comparisons of categorical values were performed with Fisher's exact two-tailed test and Chi-square test. Odds ratios were used to determine the association between various outcomes. The probability values were considered as significant at $p \leq 0.05$ cut-off value. The trial is registered at Clinicaltrials.gov: ID number NCT01061593.

Results

This study comprised 269 TB patients including 100 recruited in Mongolia and the remaining were enrolled in Ukraine. The baseline characteristics of patients in the two treatment groups were not statistically different, indicating that the outcome was not biased by sample heterogeneity (Table 1).

Lack of adverse reactions

No adverse side effects attributable to Immunoxel were seen during the entire study duration. No reactivation of TB, malaise, intolerance or allergic reactions was evident at any time during the study period. After 1 month, self-reported and physician-observed clinical symptoms appeared to improve among Immunoxel patients, while the proportion of patients with satisfactory results was considerably smaller in placebo. This subjective impression is supported by secondary end points such as an effect on body weight, fever reduction, erythrocyte sedimentation rate, leukocyte and lymphocyte counts, hemoglobin concentration and select liver function parameters as detailed below.

Effect on mycobacterial clearance

After 1 month 87 out of 132 patients (65.9%) became sputum smear negative in the Immunoxel group, whereas in placebo 32 out of 127 patients (25.2%) had sputum converted (Table 2). Both, intra- and inter-group outcomes were highly significant with relevant t-tests ($p < 0.0001$). At the baseline patients in both arms were indistinguishable in terms of their initial mean sputum scores; 1.314 ± 0.7 versus 1.328 ± 0.7 ($p = 0.87$) but their post-treatment scores differed twofold; 0.360 ± 0.6 versus 0.795 ± 0.7 ($p < 0.0001$) for Immunoxel and placebo. The two-tailed Chi-square test comparing outcomes within Immunoxel arm across four subgroups of TB patients, in other words, DS-TB, MDR-TB, XDR-TB and TB-HIV indicates that differences in conversion rates were negligible and not dependent on drug resistance or HIV status ($p = 0.34$). Contrary, the discrepancy between conversion rates in placebo was significant ($p = 0.004$) supporting the impression that higher proportion of DS-TB patients responded to ATT and those who had drug-resistant TB or TB-HIV were less responsive (Figure 1 & Table 2).

Effect on body weight

Wasting is a prominent feature of TB. The BMI can unequivocally distinguish those who are normal or cachexic based on 18.5 kg/m^2 cut-off threshold. At study entry both arms, Immunoxel and placebo, had a substantial proportion of individuals with wasting; in other words, 28/137 (20.4%) and 42/132 (31.8%) respectively. While the inter-group difference in proportions was statistically significant (p = 0.04 by Fisher's exact test), the mean absolute body weight and BMI values at baseline were similar, in other words, p = 0.62 and p = 0.62 by unpaired t-test (Table 1). After 1 month the average body weight gain in Immunoxel arm was 2 kg, from 58.8 ± 9.5 to 60.8 ± 9.7 (p = 0.07), but placebo recipients gained only 0.6 kg; 58.2 ± 9.6 to 58.8 ± 10.4 (p = 0.55). The post-treatment difference between arms in terms of weight accrual, in other words, gain or loss, was highly significant by Fisher's 2 × 2 contingency table (p < 0.0001). The proportion of patients with body weight gain versus weight loss was in favor of Immunoxel with odds ratio 8.75 in 3.84–19.95 range at 95% confidence interval (p < 0.0001). The changes in BMI mirrored absolute weight values; in Immunoxel and placebo the BMI increased from 20.21 ± 2.2 to 20.77 ± 2.3 (p = 0.04) and 20.06 ± 2.2 to 20.13 ± 3 (p = 0.92), respectively. The proportions of patients who responded favorably were 68.5 and 12.3% (p < 0.0001) for Immunoxel and placebo, respectively.

Effect on axillary body temperature

The average axillary temperatures among Immunoxel and placebo recipients were above normal 36.8°C body temperature, in other words, 37.4 ± 0.6°C and 37.7 ± 0.6°C – indicative of persistent low-grade fever. At the baseline 15 out of 41 (36.6%) patients in Immunoxel arm had normal 36.8°C body temperature, while in placebo recipients this proportion was significantly lower; 5/42 (11.9%; p = 0.01 by Fisher's test). This was actually the only baseline parameter that has been statistically different between treatment arms. After 1 month the average body temperature in Immunoxel and placebo groups had decreased to 36.8 ± 0.1°C and 37.3 ± 0.6°C (p < 0.0001 by paired t-test), the only difference being that in former group the body temperature returned back to normal, but controls remained febrile. The proportion of patients with normal bodily temperature reached 37/41 (90.2%) and 17/42 (40.5%) for Immunoxel and placebo (Fisher's test p < 0.0001). After excluding those who had normal temperature at baseline, 26 out of 41 (63.4%) Immunoxel recipients had experienced defervescence, but in controls the improvement was seen in a smaller proportion, 12/42 (28.6%) – a difference that was highly significant (p = 0.002).

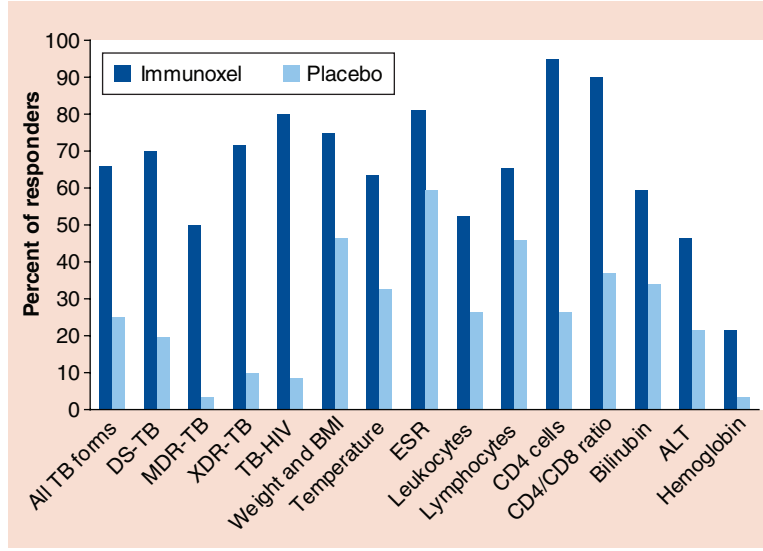

Figure 1. Proportion of patients with favorable response expressed in percentage on the Y-axis versus various end points listed on the horizontal axis. As opposed to placebo recipients those who received daily dose of Immunoxel had marked beneficial effect as described in detail in Tables 2–7.

Table 2. Sputum smear scores prior to and after 1 month of treatment in 132 Immunoxel and 127 placebo recipients.

Score	Immunoxel N (%)		Fisher's test	Placebo N (%)		Fisher's test
	Before	After		Before	After	
0	*5 (3.6)	87 (65.9)	p < 0.0001	*5 (3.8)	32 (25.2)	p < 0.0001
**0.1	4 (3)	5 (3.8)	p = 1.0	6 (4.7)	10 (7.9)	p = 0.44
1	96 (72.7)	34 (25.8)	p < 0.0001	86 (67.7)	75 (59.1)	p = 0.19
2	19 (14.3)	5 (3.8)	p = 0.005	23 (18.1)	5 (3.9)	p = 0.0005
3	13 (9.8)	1 (0.8)	p = 0.001	12 (9.4)	5 (3.9)	p = 0.13
Mean score	1.314 ± 0.7	0.360 ± 0.6	p < 0.0001	1.328 ± 0.7	0.795 ± 0.7	p < 0.0001

*Five patients in each group were acid-fast bacteria-negative at study entry and as they remained negative at study conclusion they were excluded to prevent skewing the actual conversion rate.
**The score 0.1 is assigned to cases in whom bacterial count was not sufficient to qualify as 1.

Table 3. Effect of immunotherapy and placebo on absolute body weight and BMI.

Changes in absolute body weight (kg) & BMI (kg/m²)*	Immunoxel n = 127(%)	Placebo n = 122(%)	Intergroup Fisher's test
Gained weight	95 (74.8)	57 (46.7)	p < 0.0001
Lost weight	8 (6.3)	42 (34.4)	
Same weight	24 (18.9)	23 (18.9)	p = 1.0

*The proportions of patients in terms of both, absolute body weight and BMI, were identical and were thus not listed in the Table separately.

Table 4. Changes in erythrocyte sedimentation rate and leukocyte counts prior to and after study conclusion.

Normal values	Immunoxel		Fisher's 2 × 2 test	Placebo		Fisher's 2 × 2 test
	Before	After		Before	After	
ESR normal	54/119 (45.4%)	96/119 (80.7%)	p < 0.0001	61/129 (47.3%)	70/118 (59.3%)	p = 0.07
Leukocytes	85/129 (65.9%)	104/120 (86.7%)	p = 0.0002	98/129 (71.4%)	92/117 (78.7%)	p = 0.24

Table 5. Changes in serum levels of alanine transaminase and total bilirubin after 1 month of treatment.

Liver function test outcomes at study end	ALT		Fisher's 2 × 2 test	Total bilirubin		Fisher's 2 × 2 test
	Immunoxel n = 30 (%)	Placebo n = 28 (%)		Immunoxel n = 42 (%)	Placebo n = 38 (%)	
Increased	13 (43.3)	15 (53.6)	0.60	14 (33.3)	21 (55.3)	0.07
Decreased	14 (46.7)	6 (21.4)	0.06	25 (59.6)	13 (34.2)	0.03
Same	3 (10)	7 (25)	0.17	3 (7.1)	4 (10.5)	0.70
	X^2 test p < 0.0001			X^2 test p < 0.0001		

ALT: Alanine transaminase.

Table 6. Effect of Immunoxel versus placebo on percent values of lymphocytes set at 20% threshold.

Outcome	Immunoxel n = 64 (%)	Placebo n = 61 (%)	Fisher's test
Increased*	42 (65.6)	28 (45.9)	p = 0.03
Decreased	19 (29.7)	31 (50.8)	p = 0.02
Same	3 (4.7)	2 (3.3)	p = 1.0

X^2 test p < 0.0001.
*Gain or loss is determined by cut-off value set at 20% threshold.

Table 7. Treatment effect on absolute CD4 and CD8 lymphocyte numbers in TB-HIV patients.

T cells	Immunoxel n = 22		Paired t-test	Placebo n = 20		Paired t-test
	Before	After		Before	After	
CD4 cells	309	417	p < 0.0001	306	271	p = 0.53
CD8 cells	896	865	p = 0.76	1240	746	p = 0.02
CD4/CD8	0.39	0.53	p = 0.0002	0.41	0.52	p = 0.4

Effect on TB-associated inflammation

In addition to persisting low-grade fever the patients with TB are known to have elevated erythrocyte sedimentation rate (ESR) and leukocyte counts, both of which are indicative of TB-associated inflammation. At baseline an equivalent proportion of patients had ESR levels above 20 mm/h threshold, 57.7 versus 52.3% in adjunct and placebo groups. Immunoxel decreased ESR from 21.5 ± 12 to 13.5 ± 8 mm/h (p < 0.0001), whereas in placebo the ESR has not changed, in other words, 23.8 ± 15 to 20.9 ± 16 (p = 0.23). In the immunotherapy arm the leukocytes decreased from mean 7.7 ± 2.7 to 6.98 ± 2.5 × 10^9 cells/l (p = 0.009) while among those on placebo this reduction

was not significant; 7.8 ± 2.8 to 7.4 ± 2.8 × 10^9 cells/l (p = 0.26). In terms of beneficial response rate, the proportions of responders were 91 versus 75% (p = 0.004) and 52.7 versus 26.5% (p = 0.0003) for ESR and leukocytes respectively.

Effect on anemia

Many TB patients are characterized by low hemoglobin content, which is manifested as anemia and is usually associated with poor prognosis. About half of patients in both groups, Immunoxel and control, were anemic with hemoglobin content below normal 120 g/l level; 52.3 and 47.8% (p = 0.42). After 1 month Immunoxel patients experienced the increase in hemoglobin from 121.6 ± 15.9 to 124.3 ± 15.4 g/l (p = 0.33), but in placebo the opposite trend was observed, 119.4 ± 19.7 to 116.7 ± 19.4 g/l (p = 0.13). The unpaired t-test of the outcome shows that inter-group difference is significant (p = 0.007), which is also attributable to change in the proportion of anemic patients, 36.6 versus 50.6%, respectively.

Effect on liver function

TB drugs are known to adversely affect liver function. We have evaluated whether Immunoxel affects in any way the drug-induced hepatotoxicity by measuring alanine transaminase (ALT) and total bilirubin levels. In Immunoxel arm the difference between baseline and post-treatment mean ALT values expressed in mM/h*l was 0.243 ± 0.11 versus 0.274 ± 0.13, which by paired t-test was not significant (p = 0.23). In placebo recipients, however, the mean ALT values have increased significantly within 1 month, in other words, from 0.289 ± 0.2 to 0.441 ± 0.43 (p = 0.05). The total bilirubin values (g/L) in Immunoxel arm declined from 12.6 ± 3.8 to 11.4 ± 3.4 (p = 0.1) while in placebo they remained unchanged 11.1 ± 2.6 versus 11.04 ± 2.5 (p = 0.32). The proportions of patients in two treatment groups who had either decreased, increased or same bilirubin levels at study conclusion had an inverted pattern, which was statistically significant (p < 0.0001). Similar tendency was observed with ALT; higher proportion of patients on placebo had increased ALT values, but those on Immunoxel were more likely to have decreased liver enzyme levels. Chi-square analysis across three types of response indicates that the difference between two treatment arms was highly significant (p < 0.0001).

Effect on lymphocyte counts

TB patients display diminished population of lymphocytes, often below 20% normal threshold. In our patients the average baseline figures were 23.5 ± 9% and 23.1 ± 9.5% (p = 0.78) and the proportion of patients with <20% lymphocytes was 38.2 versus 35.9% (p = 0.86) for Immunoxel and placebo, respectively. After 1 month Immunoxel patients had their lymphocytes increased to 26.2 ± 11% (p = 0.09), but in controls they declined to 21.4 ± 7% (p = 0.23). Both outcomes were not statistically significant but this trend could be further seen in a breakdown analysis of responders versus nonresponders. Table 1 shows that 65.6% of Immunoxel recipients versus 45.9% in placebo arm were gaining lymphocytes (p = 0.03), while 29.7 versus 50.8% (p = 0.02) were losing, with a negligible proportion 4.7 versus 3.3% had stable lymphocytes counts.

Effect on CD4 & CD8 T-lymphocyte counts among HIV+ TB patients

The hallmark of HIV infection is the depletion of CD4 T cells. In both, Immunoxel and placebo arms, patients with HIV had initially low absolute numbers, but there was no difference between groups (p = 0.96). After 1 month, however, among those who received Immunoxel the absolute CD4 cell count had risen significantly, from 309 ± 156 to 417 ± 159 (p < 0.0001), but decreased in control patients from 306 ± 228 to 271 ± 211 (p = 0.53). Although the decrease in control patients was not statistically significant there was also a difference in terms of response rate. In Immunoxel group, all patients, except one, had improved CD4 cell counts (95%) while in placebo only a quarter (26.3%) had experienced the increase. A similar trend was seen with the percentage of CD4 lymphocytes albeit the p-values were slightly different, in other words, 0.008 and 0.39, respectively. Patients on placebo had initially higher absolute number of CD8 cells than those in Immunoxel arm, in other words, 1240 ± 883 versus 896 ± 487 (p = 0.13). At study conclusion CD8 cell numbers decreased to 746 ± 598 (p = 0.02) and 864 ± 278 (p = 0.76), respectively. Another important parameter that helps to assess the disease progression is CD4/CD8 cells ratio; those who have higher ratio tend to have better prognosis. While CD4/CD8 ratios had increased to approximately same levels in both groups, the statistical significance has been reached in the Immunoxel arm only (p = 0.0002).

Discussion

TB is out of control in developing countries due to increasing poverty, drug-resistant TB and HIV co-infection [1]. Many previously reported immune interventions including inhaled IFN-γ, vitamin D, corticosteroids, thalidomide and various cytokine or anticytokine regimens showed some improvements, but more often results were inconsistent and occasionally associated with deleterious side effects [11,15–17]. In view of such situation it is clear that safer and better

immune therapies are needed. The results of this randomized, placebo-controlled, double-blind, Phase III study in a representative population of TB patients in two countries with distinct ethnic backgrounds, indicates that when TB drugs are combined with Immunoxel honey lozenges this combination produced faster clearance of *M. tuberculosis* at a significantly higher rate than in placebo arm. After 1 month 65.9% of Immunoxel recipients became sputum smear negative, whereas in placebo only 25.2% sputum converted. This response rate, which occurred within such a short period of time, has rarely been seen in chemotherapy trials [2]. In addition there is a twofold difference in mean study-end sputum score, in other words, 0.360 versus 0.795, indicating that Immunoxel helped to achieve lower bacterial load among those who had not yet converted. These findings support our twenty clinical studies involving about 1500 individuals with DS-TB, MDR-TB, XDR-TB and TB-HIV, including the recent Phase II trial, which evaluated the honey lozenge formulation [11–14].

It is well known that fever, cachexia, leukocytosis, lymphopenia, elevated ESR and low hemoglobin content are requisite parameters to gauge the severity of TB [18,19]. The return of these indices to normal levels is an indication of disease control and a good correlation has been found with sputum conversion [19]. Tuberculous pyrexia is an independent risk factor associated with poor prognosis; inhaled corticosteroids are known to produce fast and reliable defervescence supporting the role of anti-inflammatory intervention in TB patients [16]. Immunoxel helps to reduce the body temperature in a higher proportion of patients, in other words, 63.4 versus 28.6% in placebo – a ratio that matches sputum conversion rate. Consumption is a term historically synonymous with tuberculosis and represents poorly manageable wasting syndrome associated with malnutrition and chronic inflammation [20]. The remarkable aspect of our immunotherapy is body weight gain, which was a threefold higher than in controls. None of our patients received weight-boosting supplements, thus ruling out the role of nutritional intervention. In general, our experience with Immunoxel and unrelated TB immunotherapies indicates that a strong relationship exists between reversal of cachexia and immune intervention. The odds ratio analysis supports this conjecture (OR: 8.75; 95% CI: 3.84–19.95; $p < 0.0001$). The notion that weight loss in TB is associated with inflammation is supported by the potent anti-inflammatory activity of Immunoxel. Two simple biomarkers associated with inflammation and common in TB are leukocytosis and elevated ESR [18]. The effective TB therapy has been shown to normalize these markers [19]. Immunoxel reduced the ESR and leukocyte counts more efficiently than ATT alone. Anemia is another common manifestation of pulmonary TB and HIV. The precise cause of anemia is not clear, but inflammation and iron deficiency are thought to be the main culprits [21]. As our patients were not receiving iron supplementation, we believe that the increase in hemoglobin content relates to anti-inflammatory property of Immunoxel. The reversal of lymphopenia in TB, including increase in CD4 lymphocytes among HIV+ patients is another beneficial outcome seen in this and prior Immunoxel trials. Finally, as judged by ALT and bilirubin assays, Immunoxel counteracts drug-induced hepatotoxicity – an important factor that affects negatively compliance and success of TB chemotherapy.

What is the mechanism of Immunoxel action? It is unlikely that Immunoxel affects directly mycobacterial replication; *in vitro* studies have shown that the growth of reference TB strains H32 and H37Rv was not affected by physiologically relevant doses [13]. Immunoxel targets the host rather than *M. tuberculosis* since patients with drug-resistant TB and etiologically unrelated diseases, in other words, HIV, hepatitis, flu, cancer, and a range of autoimmune diseases were equally responsive to this immune intervention. TB is a disease whereby pulmonary tissues harboring mycobacteria are constantly assaulted by the host's immune system, creating chronic inflammation and ensuing pulmonary damage [11,15]. Therefore, immune therapies should be aimed at downplaying rather than exacerbating an already intense immune response. We know that Immunoxel can efficiently reduce pro-inflammatory cytokines [22]. However, it is not sufficient to just suppress inflammation since various anti-inflammatory agents produced contradictory outcomes in TB [23–26]. We do not know how the down-regulation of inflammation correlates with bacterial clearance; the mechanism of action of Immunoxel is unknown and further studies addressing this question needs to be carried out.

How do our results compare to other botanical products? Many medicinal plants are being evaluated for use against TB, but they are mostly in early stages of development. To the best of our knowledge, clinical results from only three plant-derived preparations have been reported in peer-reviewed literature. The oldest known is Umckaloabo – a traditional South African medicine from the roots of *Pelargonium sidoides* – used to treat TB and respiratory tract infections since 19th century [27]. Another report concerns inhaled tea tree oil from Australian *Melaleuca alternifolia*, which produced beneficial outcome in one case of advanced TB [28]. Finally, a multiherbal water-infusion cocktail based on Russian folk medicine has been reported to

shorten TB treatment to 6.4 months, instead of 8.6 months in controls [29]. For other herbal formulations, commonly used within traditional medicine practice in many parts of the world, the evidence is mostly anecdotal, lacking well-designed clinical studies [30].

Our study had several limitations. One limitation is that study concluded after 1 month without providing information beyond this time-point, for example, relapse rate in Immunoxel arm. We had extensive long-term follow-up experience encompassing 1500 patients, indicating that such relapses are rare, in other words, no more than one in 100 over 12 months. Our budget was not large enough to carry out the longitudinal study; our priority was to establish that treatment regimen can be shortened. The choice of the time-span was deliberate as we knew *a priori* that the difference between immunotherapy and placebo will become apparent within such a short time period. Second, we have relied on sputum smear conversion by microscopy rather than culture-based method. Again, based on our own experience of using either of the methods and published evidence, both criteria are equally valid in assessing treatment efficacy, difference being only 1–2%, but former method is certainly easier, faster and less costly, making it more suitable in countries with limited infrastructure [31]. Third, our sample size was limited in regard to some of the measured parameters as complete lab analysis was not available at every site and budget restraints prevented us establishing centralized lab facility. Nevertheless, whatever was available was sufficient in every instance to reach respectable p-values to support our conclusions. Fourth, it may appear at first glance that we had very relaxed inclusion criteria, but again it was our conscious intention to have real-life population sample without variance from the natural occurrence of TB in its various manifestations.

In conclusion, we believe strongly that Immunoxel holds promise in improving treatment outcome in drug-refractory TB. Immunoxel is available over-the-counter in Ukraine, Mongolia and five other countries as an immune supplement, but the current thinking is geared toward developing TB drugs to suppress mycobacteria, but interventions targeting the host have been somewhat neglected. Immunomodulators, especially those that do not require a prescription, are dismissed off-hand and not taken seriously despite superior performance. The global emergence of drug-resistant TB and TB-HIV – a problem that is unlikely to go away by itself – concerns greatly TB caregivers and policymakers [1,2]. This ongoing public-health threat may prompt a shift from the current paradigm and may result in a wider acceptance of immunotherapeutic approaches as an adjunct to TB treatment regimens.

This Phase III trial culminates ours and independent Ukrainian TB doctors' effort, first reported back in 1999 [13], which since had evaluated Immunoxel for a multitude of clinical indications, including DS-TB, MDR-TB, XDR-TB and TB-HIV [11–14,22,32–47]. All these studies reported beneficial outcome with added benefit of drastically reducing treatment duration to as short as 1 month. However, the original liquid formula known as Dzherelo, later replaced by improved version, Immunoxel, was not convenient for use and efforts were made by us to identify easy-to-use solid formulations, one of which was honey-based lozenge that had the best performance, perhaps due to synergy with historically known benefit of honey as TB remedy [14]. Our findings derived from use of solid formulation support 20 previous clinical trials of Immunoxel showing shortened duration, safety and potent anti-inflammatory property supported by better effect on well-being, superior healing of pulmonary lesions, body weight gain, defervescence, leukocytosis, lymphopenia, better ESR, hemoglobin and reduced hepatotoxicity caused by TB drugs. These beneficial effects were statistically more significant than in ATT alone arm (Figure 1). The authors hope that Immunoxel advantages will be not dismissed off-hand and adequate policy changes will be implemented to embrace this and other safe and effective immunotherapies we and others have worked on in the past [48].

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Ethical conduct of research

The authors have obtained the Institutional Review Board approvals and have followed the principles of the Declaration of Helsinki. The participants in the trial have provided informed consent, participated in this study voluntarily and were free to withdraw from this study at any time. This study is registered with the ClinicalTrials.gov under identifier NCT01222338.

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Executive summary

- Herbal phytoconcentrate, Immunoxel, formulated into sublingual honey lozenge has been administered for 1 month in a double-blind, placebo-controlled, 1:1 randomized Phase III clinical trial in 269 patients with pulmonary TB in two high-burden countries, Mongolia and Ukraine.
- The primary end point was sputum smear conversion by microscopy.
- Baseline characteristics of patients in Immunoxel and placebo were similar; 102 patients versus 106 had DS-TB; 28 versus 20 MDR-TB; 7 versus 7 XDR-TB; and 21 versus 20 had TB-HIV.
- The once-daily administration of Immunoxel honey lozenge along with first- or second-line TB drugs resulted in the clearance of Mycobacterium tuberculosis in sputum smears in 87 out of 132 (65.9%) of Immunoxel recipients versus 32 out of 127 (25.2%) in placebo group.
- Sputum conversion occurred very fast – only 1 month of treatment was needed.
- Sputum clearance produced by Immunoxel was equally effective across all forms of TB, while in controls only drug susceptible TB (DS-TB) had the expected response rate.
- Differences in gender, age and body weight had no influence on conversion rates.
- Immunoxel reversed TB-associated wasting; the average weight gain was 2 kg versus 0.6 kg of placebo recipients. Weight gain was seen in 68.5 versus 12.3% in two treatments arms, respectively.
- Immunoxel eliminated TB-associated fever in 63.4% patients versus 28.6% of placebo recipients.
- Immunoxel demonstrated marked anti-inflammatory effect as judged by ESR and leukocyte counts; the proportions of responders were 91 versus 75% and 52.7 versus 26.5% for ESR and leukocytes, respectively.
- Immunoxel is safe; it has not produced any adverse effects or caused reactivation of TB.
- Immunoxel is effective and affordable, easy to administer and made from renewable sources.

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