PREVENTION BY *PSEUDOMONAS PUTIDA* OF THE BENZENE ACCUMULATION IN TUMOR AND NORMAL TISSUES (PILOT STUDY)

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ПРЕДОТВРАЩЕНИЕ НАКОПЛЕНИЯ БЕНЗОЛА ОПУХОЛЕВЫМИ И НОРМАЛЬНЫМИ ТКАНЯМИ С ПОМОЩЬЮ *PSEUDOMONAS PUTIDA*

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The ability of *Pseudomonas putida* to metabolize benzene accumulated in normal and tumor human tissues was tested *in vitro*. The metabolic degradation of benzene after incubation *in vitro* of normal and tumor tissues with benzene at 25 °C for 24 h was about 80 and 50% respectively, the rest of benzene has been accumulated. When incubation with benzene occurred in presence of *P. putida* the final metabolization value reached almost 100%. It was suggested that *P. putida* could be used as a potential treatment agent in the modulation of toxic states caused by benzene accumulation.
***Key Words*: benzene, *Pseudomonas putida*, human tissue, metabolic degradation, GC-MS.**

В работе исследована способность бактерии *Pseudomonas putida* метаболизировать бензол, аккумулированный нормальными и опухолевыми тканями человека. Метаболическая деградация бензола после инкубации с нормальными и опухолевыми тканями при температуре 25 °С в течение 24 ч составила 80 и 50% соответственно, а оставшийся бензол был аккумулирован. Если такая инкубация проводилась в присутствии *P. putida*, то конечный уровень деградации бензола составлял 100%. Таким образом, полученные данные свидетельствуют о потенциальной возможности использования *P. putida* с целью снижения токсических проявлений накопления бензола в организме человека.
***Ключевые слова*: бензол, *Pseudomonas putida,* ткани человека, метаболическая деградация, ГХМС.**

The pollution from benzene particularly in urban areas brings to hard problems of social hygiene and safety because human organism can accumulate this compounds from the environment mainly by the respiratory way. The benzene which penetrates into the human body partly is excreted with urine or respiration, partly is metabolized by oxidase and sulphatase enzymes [1], partly (20–40%) is accumulated [2] as recently we have shown [3,4]. The accumulation is favoured by the lypophylic nature of benzene and by the presence in human body of organs with high lipidic content. Other reasons of increased assumption of benzene are the high values of environmental concentration, the cigarette smoke, the physical activity and the diet [5, 6]. Individuals exerting physical activity adsorb by about 60–80% more benzene than the ones who do not. The particular attention to benzene is due to its diffusion on the ecosystem where it attains relevant concentration levels especially bound to the worldwide use of green fuel and to the unperfect functioning of vehicle engines; it was statistically demonstrated that a correlation exists between benzene toxicity and concentration and tumor origin [7–13]. In this paper, we face the problem of the accumulation of benzene in human organs looking for contrasting treatments: the one that we propose is based on *Pseudomonas putida,* a gram negative aerobic microorganism able to degrade benzene and so potentially to act as a detoxifying agent.

Materials. Benzene, toluene and *n*–pentane were from Carlo Erba (Milan, Italy). The reagents were of analytical–reagent grade with purity ≥ 99.5%. Headspace vials (2 ml) were sealed with aluminum caps with tear–out center disks an Teflon–faced septa were from Supelco (Milan, Italy). Analytical balance mod. E42 was from Gibertini (Italy). The tissue samples from six patients were supplied from "Policlinico Umberto I" (University of Rome) and from Urological Clinic of University "La Sapienza" of Rome and stored at –20 °C in N_2 atmosphere. The samples were given with the agreement of the Ethical Committee. *P. putida* culture was supplied from Department of Genetics and Biology of Microorganisms (University of Milan).

Equipment. Gas Chromatography–Mass Spectrometry (GC–MS) analyses were carried out on a Hewlett–Packard (HP, Rome, Italy) gas chromatograph, mod. 5890 series II, connected to a HP 5970B mass selective detector equipped with a PC HP 9000 (HP). The gas chromatograph was equipped with a fused–silica SPB–5 capillary column, 30 m length, 200 µm I.D. and d_f= 0.25 µm, by Chrompack (Italy).

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Abbreviation used: GC–MS — gas chromatography–mass spectrometry.

Liver and kidney samples analysis. Six patients with malignant tumors, where four affected by renal tumor and two by liver tumor, were included in this study. Both the histologically normal part and tumor of the kidney and liver were tested. Benzene standard solution (1–3 μl, 0.6 $\mu g \cdot \mu l^{-1}$) was used for each tissue sample. Tissue samples (60 mg per vial, each) were put into the vials and benzene (1.2 $\mu g \cdot g^{-1}$, 1.4 $\mu g \cdot g^{-1}$ or 1.6 $\mu g \cdot g^{-1}$) alone or with 25 μl of *P.putida* culture ($0.5 \cdot 10^8$ or $1 \cdot 10^8$ cells/ml) were added. All vials were then closed with an aluminum cap containing a polytetrafluoroethylene (PTFE) coated rubber septum and then incubation at 25 °C for a time of 0, 2, 4, 8 and 24 h. After incubation toluene standard solution (0.30 $\mu g \cdot g^{-1}$), as internal standard, was added to each vial, which was kept at 80 °C for 30 min for the conditioning before the GC–MS analysis. GC–MS analysis was carried out by manually injecting 50 μl of the vial headspace from each sample by means of a 100 μl Hamilton gas tight syringe using the operating conditions described below.

HSGC–MS analysis and quantification. Experimental conditions for GC–MS analysis were the following: the fused–silica capillary column was kept at 50 °C for 4 min and heated up to 200 °C at a programmed temperature of 10 °C/min and maintained for 5 min. The injector temperature was 230 °C and transfer line was kept heated at 200 °C. The injections were made in splitless mode (30 s delay before opening the splitter) using helium as carrier gas at u = 25 $cm \cdot s^{-1}$. Headspace analysis of the samples was performed in selected ion monitoring (SIM) mode [ions m/z 78 for benzene and m/z 92 for toluene (i.s.)]. The concentration of benzene was obtained by a calibration graph recorded by plotting the ratio area(m/z = 78)/area(m/z = 92, i.s.) *vs* the benzene concentration. The investigated concentrations were: $15 \cdot 10^{-3}$, $100 \cdot 10^{-3}$, $500 \cdot 10^{-3}$, 1.0 and $10 \cdot 10^{-1}$ $\mu g \cdot g^{-1}$. Detailed information on the experimental conditions was reported earlier [3].

In a recent paper we evidenced that human tissues can accumulate benzene, more the tumor than normal ones [3, 4]. In Table 1 the results obtained on degrading *in vitro* benzene accumulated in different tissue samples incubated with 1.2 $\mu g \cdot g^{-1}$ of benzene for 24 h are presented. These results allow us to draw two considerations: the first — behavior of the normal tissues of the two different organs (kidney, liver) is very similar: really, both can metabolize almost 80% of benzene within 24 h, and the second — this process reached in tumor at the most 50%, so showing a decreased enzymatic activity. It is reasonable to admit that unmetabolized benzene is daily accumulated in the tissues.

From this point of view, it looks reasonable to testify whether *P. putida* was able to increase the degree of benzene metabolization *in vitro* both in normal and tumor tissues. It is known that *P. putida* is very mobile due to polar flagellum and it was only occasionally insulated from human pathological materials, so it is not assumed as responsible of pathologies [14]. Table 2 demonstrates that independently on the kind of the considered tissue after incubation with benzene (1.6 $\mu g \cdot g^{-1}$) for 24 h when *P. putida* (25 μl, $0.5 \cdot 10^8$ cells/ml) was presented in the

Table 1. Metabolized benzene as percentage of the present amount within 24 h by the samples of human tissues incubated with benzene (1.2 $\mu g \cdot g^{-1}$) at 25 °C

Incubation time (h)	Metabolized benzene					
	Patient 1		Patient 2		Patient 3	
	Liver N	Liver T	Kidney N	Kidney T	Kidney N	Kidney T
0	0	0	0	0	0	0
2	35.0 ± 2.0	32.0 ± 2.0	36.0 ± 2.1	31.0 ± 2.0	35.0 ± 2.0	32.0 ± 2.1
4	55.0 ± 1.9	46.0 ± 2.0	54.0 ± 2.0	45.0 ± 1.9	57.0 ± 1.9	45.0 ± 2.0
8	62.0 ± 1.8	49.0 ± 2.1	64.0 ± 2.0	48.0 ± 1.8	66.0 ± 1.7	47.0 ± 1.8
24	81.0 ± 1.9	51.0 ± 2.0	78.0 ± 1.8	50.0 ± 2.0	80.0 ± 2.1	49.0 ± 2.0

Tissue samples: the histologically normal part (kidney N, liver N) and the tumor one (kidney T, liver T).

Table 2. Metabolized benzene as percentage of the present amount within 24 h by the samples of human tissues incubated with benzene (1.6 $\mu g \cdot g^{-1}$) and *P. putida* (25 μl, $1 \cdot 10^8$ cells/ml) at 25 °C

Incubation time (h)	Metabolized benzene					
	Patient 4		Patient 5		Patient 6	
	Kidney N	Kidney T	Liver N	Liver T	Kidney N	Kidney T
0	0	0	0	0	0	0
2	7.0 ± 1.9	3.0 ± 1.8	8.0 ± 2.0	4.0 ± 1.8	8.0 ± 2.0	3.0 ± 1.9
4	15.0 ± 1.9	7.0 ± 2.0	18.0 ± 1.9	9.0 ± 2.0	17.0 ± 2.1	6.0 ± 1.9
8	57.0 ± 2.0	26.0 ± 1.9	59.0 ± 2.0	29.0 ± 1.9	56.0 ± 1.8	27.0 ± 2.0
24	100.0 ± 2.1	100.0 ± 2.0	100.0 ± 1.9	100.0 ± 2.0	100.0 ± 1.9	100.0 ± 2.1

Tissue samples: the histologically normal part (kidney N, liver N) and the tumor one (kidney T, liver T).

incubation step, benzene is fully metabolized. A different behavior looks evident after a careful analysis of the reported results. Really the metabolization of benzene in the case of the tumor tissue occurs initially more slowly than in the case of the normal tissue. It can be hypothized that *P. putida* needs a certain time to be adapted to a new system and this time being longer for tumor than for normal tissue. Anyway, at the end of the metabolization in both cases approximately about 100% of benzene degradation is observed.

We passed to explore the influence of *P. putida* concentrations: Fig. 1 shows the results obtained on the samples of human tissue, incubated for 24 h with benzene (1.4 $\mu g \cdot g^{-1}$) and *P. putida* cells (25 μl, $0.5 \cdot 10^8$ cells/ml) — half of that one used for the data in the cases of Table 2 and Fig. 2. The diagram shows that the metabolization depends on the duration of incubation. The behavior is nearly the same for both normal tissues and tumor. *P. putida* at this concentration resulted in the slightly less differences (about 1–3%) of the benzene metabolization degree in tumor in comparison with those observed when double *P. putida* concentration was used. For instance, the benzene

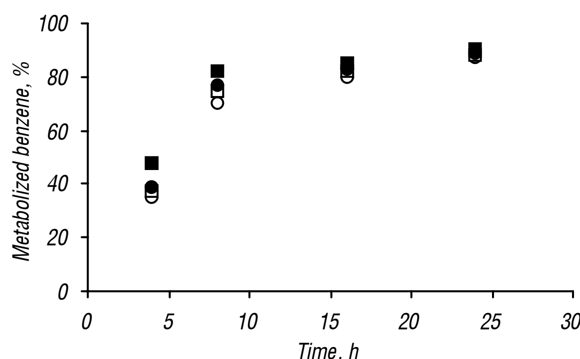

Fig. 1. Time–course of benzene metabolization by human tissues (all investigated samples) incubated with benzene (1.4 $\mu g \cdot g^{-1}$) and *P. putida* (25 μl, $0.5 \cdot 10^8$ cells/ml).
Symbols: ■ liver N; ● kidney N; □ liver T; ○ kidney T; N — normal tissue; T — tumor tissue

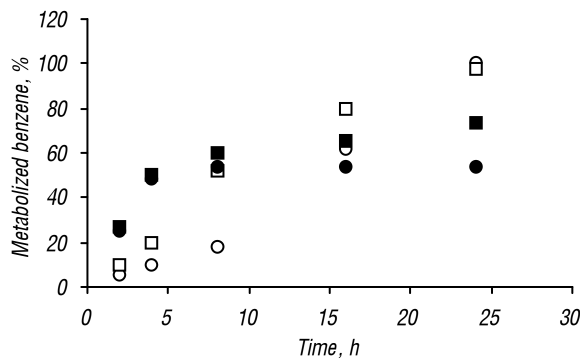

Fig. 2. Time–course of benzene metabolization by human tissues (patients 4–6). Symbols: ■ liver N + benzene (1.4 mg·g^{-1}); ● liver N + benzene (1.4 mg·g^{-1}) + *P. putida* (25 ml, 1 · 10^8 cells/ml); □ kidney T + benzene (1.4 mg·g^{-1}); ○ kidney T + benzene (1.4 mg·g^{-1}) + *P. putida* (25 ml, 1 · 10^8 cells/ml); N — normal tissue; T — tumor tissue

metabolized by the human tissues passes from 100% to less than 90% on halving *P. putida* concentration after 24 h of incubation. Comparison of data presented in Fig. 1, where initial rate looks higher, with Fig. 2 can be concluded that a lower *P. putida* concentration seems to be adapted more rapidly to the new matrix, so exerting its metabolizing action more quickly.

In conclusion, the reported results show that *P. putida,* due to its action on the metabolism of benzene accumulated in human organs, is a potential modulating agent in the case of toxic states caused by benzene. Its action, evaluated *in vitro* test, is efficient even when human tumor tissues are considered. In this case the rate of detoxification that is of the order of hours depends on *P. putida* concentration.

REFERENCES

1. **Smith MT, Yager JW, Steinmetz KM, Eastmond DA.** Peroxidase-dependent metabolism of benzene's phenolic metabolites and its potential role in benzene toxicity and carcinogenicity. Environ Health Persp 1989; **82**: 23–9.

2. **Susten A, Dames B, Burg J, Niemeir R.** Percutaneos penetration of benzene in hairless mice: an estimate of dermal adsorption during tire building operation. Am J Ind Med 1985; **7**: 325.

3. **Russo MV, Campanella L.** Static headspace analysis by GC-MS (in SIM mode) to determine the benzene in human tissues. Anal Lett 2001; **34:** 883–91.

4. **Campanella L, Castaldi M, Grossi R, Pigliucci G, Russo MV.** Benzene accumulato negli organi umani. Determinazione con nuovi metodi analitici. La Chimica e l'Industria 2001; **83**: 1–7.

5. **Pezzagno G, Imbriani M.** Factors affecting the biologic levels of benzene in humans. In: Advances in occupational medicine and rehabilitation. Fondaz S., ed. Italy: Maugeri Edizioni 1995; **1**: 27–38.

6. **Pezzagno G.** Rischio da benzene. Italy: La Goliardica Pavese, 1989.

7. **Sammett D, Lee EW, Kocsis JJ, Snyder R.** Partial hepatectomy reduces both metabolism of benzene. J Toxicol Environ Health 1979; **5**: 782–92.

8. **Glatt H, Witz G.** Studies on the induction of gene mutations in bacterial and mammalian cells by ring-opened benzene metabolites trans,trans-muconaldehyde and trans,trans-muconic acid. Mutagenesis 1990; **5**: 263–6.

9. **Snyder R, Wits G, Goldstein BD.** The toxicology of benzene. Environ Health Persp 1993; **100**: 293–306.

10. **Cirasino L, Invernizzi R.** Hematological changes in occupational exposure to benzene. In: Advances in occupational medicine and rehabilitation. Fondaz S., ed. Italy: Maugeri Edizioni 1995; **1**: 121–34.

11. **Jakobsson R, Ahlbom A, Bellander T, Lundberg I.** Acute myeloid leukemia among station attendants. Arch Environ Health 1993; **48**: 255–9.

12. **Nilsson RI, Nordlinder RC, Tagesson C, Walles S, Jarvholm BG.** Genotoxic effects in workers exposed to low levels of benzene from gasoline. Am J Ind Med 1996; **30**: 317–24.

13. **Campanella L.** Analytical chemical considerations on tumor genesis. Exp Oncol 2001; **23:** 76–7.

14. **Pasquinelli F.** Diagnostica e tecniche di laboratorio. Italy: Rossini Editrice 1981; **2**.