ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA
VOL. XXIII, N 1, 12 SECTIO DDD 2010

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Effect of tirapazamine on oxidative stress and metabolic parameters in skeletal muscle of rats treated with classic anticancer drugs

Ocena wpływu tirapazaminy na wykładniki stresu oksydacyjnego i metabolizmu komórkowego w mięśniach szkieletowych szczurów otrzymujących wybrane leki przeciwnowotworowe

A large percentage of solid tumours contain low oxygenated cells compared to healthy tissues. These cells do not respond to ionising radiation and certain chemotherapeutic drugs and, as a result, recurrence of malignancies may appear. Therefore, resistance of tumour hypoxic cells is a principal problem in cancer treatment and three general strategies to resolve treatment issues have been developed. Firstly, chemical sensitizers to selectively increase the radiation sensitivity of the hypoxic cells are widely investigated. Secondly, a lot of effort has been made to increase tumour oxygenation which is a critical factor influencing radiation response. Thirdly, current research has underlined the importance of hypoxic cell citotoxin that can selectively kill tumour cells. Tirapazamine (TPZ) is the first hypoxia-selective agent introduced to the clinical trials [2]. So far, pre-clinical and clinical studies with TPZ have shown that the drug is highly effective in killing hypoxic cells, 50–200 times more successfully than well-oxygenated cells, depending on cell line [1]. TPZ is a potentially non-toxic compound that is bioreductively activated via one electron reduction to reactive-drug free radical with cytotoxic properties. Under moderate to very low oxygen tension, TPZ radicals cause single and double-strand DNA breaks and consequently chromosome aberration what leads to cell killing. Under normoxic or hyperoxic conditions, TPZ radicals are back-oxidised to the parent molecule with the concomitant formation of superoxide radical ($O_2^{\bullet -}$), thereby TPZ radical induced damage is to a large extent prevented. Therefore, TPZ is a promising chemotherapeutic compound to exploit showing anti-tumour activity in combination with classical chemotherapy as well as radiotherapy. So far, studies involving TPZ combined with doxorubicin (DOX), cisplatin (CP) or 5-fluorouracil (FU) have been conducted [4].

The analysis of TPZ red-ox transformation indicates that $O_2^{\bullet -}$ is produced in normal cells at a higher level of pO2. This process is catalysed by NAD(P)H dependent enzymes and when

one-electron reduction is periodically and persistently repeated it could lead to red-ox equilibrium disorders or even oxidative stress. Thus, it is expected that TPZ when administered with cell red-ox status changing drugs potentiates symptoms by additive activity. There is strong evidence that doxorubicin and cisplatin can change red-ox status in cells [8, 10]. NAD(P)H/NAD(P) ratio is very important in hydrocarbons, lipids and proteins metabolism; besides it is well known that some lipid peroxidation products – oxidative stress markers – can inhibit metabolic processes, e.g. Krebs cycle [7]. The aim of the present study is to evaluate the extend of lipid peroxidation and metabolic parameters in skeletal muscle of rats treated with classic anticancer drugs (doxorubicin, cisplatin, 5-fluorouracil) when combined with TPZ.

MATERIAL AND METHODS

The experiment was approved by Local Bioethical Commission of Medical University of Lublin. Male Wistar rats strain were obtained from Breeding Rats Brwinów/Warsaw Animals with initial body weight of 160–195g were maintained in stable conditions at 22˚C with a 12-h light/dark cycle and given standardized granulated fodder LSM. The experiment was conducted at the Central Animals Unit of Medical University of Lublin being under supervision of Veterinary Inspectorate in Puławy. The rats were treated (i.p.) with tirapazamine, synthesised by ADVANCED TECH. & IND. CO., LTD., China in two doses (5 and 10 mg/kg b.w.), 2h before administration of 1.8 mg/kg b.w. of doxorubicin (Ebewe Arzneimittel Ges. MBH, Austria), 2 mg/kg b.w of cisplatin (Cefarm, Poland) or 10 mg/kg b.w. of 5-fluorouracil. All tested drugs were administered once a week over a period of 6 weeks and a week after the last dose the skeletal muscle samples were taken to be tasted. To avoid the contribution of the red blood cells remaining in the skeletal muscle to the measured parameters, the tissue samples were washed with 20 ml of saline then placed in liquid nitrogen and stored at −75 °C until homogenisation procedure. Tissue samples were homogenized in 20 mM phosphate buffer (pH 7.4; proportions: 0.5g of tissue and 2 cm^3 of buffer) in homogenizer with Teflon piston (5 minutes at 4000 rpm). Then, homogenates were centrifuged at 14 000 rpm at 4˚C for 20 minutes. The concentration of measured parameters were tested at the microplate reader Power Wave xs (BioTek USA). Lipid peroxidation parameters were assayed with a colorimetric kit (Biotech LPO-586TM (OxisResearchTM, USA) as a reaction with malondialdehyde and 4-hydroxyalkenals. Triglycerides and glucose concentrations were measured using commercial diagnostic kits (Cormay, Poland). Bradford reagent (Sigma, USA) was used to measure the concentration of protein in skeletal muscle homogenates. The procedure is based on the formation of a complex between Bradford reagent and proteins in solution. The protein-reagent complex causes a shift in the absorption maximum of the reagent from 465 to 595 nm. The linear concentration range is 0.1–1.4mg/ml of protein with Bovine serum albumin used to prepare the standard for comparison. When needed phosphate buffer was used for diluting. The whole procedure was conducted as directed by the manufacturer and then a standard curve of absorbance versus micrograms protein was prepared to determine concentrations of original samples from the amount of protein, volume/sample, and dilution factor, if any.

RESULTS

The concentration of lipids peroxidation parameters in skeletal muscle samples was significantly lower in rats receiving DOX with TPZ versus DOX. Regardless of TPZ dose, MDA+4HNE level about 5 times lower than in DOX group was observed. The differences were not significant in triglycerides, glucose and proteins levels as DOX and DOX+TPZ groups were compared.

There were also insignificant variations in lipids peroxidation products concentrations and protein levels between CP and CP+TPZ groups. Looking at the triglycerides levels, these results showed significantly lower levels in rats treated with CP and TPZ versus CP only. The triglycerides concentrations are approximately 50% of the CP group value. Similar changes were observed in these group as glucose levels were assessed. Combination of CP and TPZ lowered the glucose levels

Table 1. The levels of chosen parameters in skeletal muscle of rats treated with doxorubicin and tirapazamine

Group	MDA=4HNE		Triglycerides		Glucose		Proteins	
	(μM)	N	(g/l)	N	(g/l)	N	(g/l)	N
DOX	8.22±4.42	6	2.89±1.03	6	0.744±0.253	6	10.79±1.17	6
DOX+5TPZ	1.55±0.22*	6	3.59±2.23	6	0.723±0.175	6	10.06±1.56	6
DOX+10TPZ	1.50±0.39*	6	2.38±0.57	6	0.670±0.102	6	9.90±2.09	6

*p≤0.05; DOX – doxorubicin; TPZ – tirapazamine

Table 2. The levels of chosen parameters in skeletal muscle of rats treated with cisplatin and tirapazamine

Group	MDA=4HNE		Triglycerides		Glucose		Proteins	
	(μM)	N	(g/l)	N	(g/l)	N	(g/l)	N
CP	2.47±0.55	6	7.37±1.28	6	0.835±0.131	6	11.98±1.70	6
CP+5TPZ	1.93±0.57	5	2.38±2.05*	5	0.605±0.080*	5	11.01±1.65	5
CP+10TPZ	2.63±0.50	6	3.71±1.40*	5	0.723±0.171	6	13.20±3.50	6

*p≤0.05; CP – cisplatin; TPZ – tirapazamine

Table 3. The levels of chosen parameters in skeletal muscle of rats treated with 5-fluorouracil and tirapazamine

Group	MDA=4HNE		Triglycerides		Glucose		Proteins	
	(μM)	N	(g/l)	N	(g/l)	N	(g/l)	N
FU	4.89±1.42	6	3.83±0.97	6	0.688±0.106	6	12.92±0.89	6
FU+5TPZ	1.50±0.27*	5	2.65±1.32	5	0.617±0.222	5	11.51±3.60	5
FU+10TPZ	3.09±1.38	5	2.82±1.35	5	0.822±0.110	4	14.54±0.44	5

*p≤0.05; FU – 5-fluorouracil; TPZ – tirapazamine

compared to CP only group; however, the only significant difference was noticed in rats treated with CP and TPZ lower dose. There were no significant changes in protein levels of CP rats as well as CP + TPZ groups.

The level of MDA+4HNE was significantly lower in rats treated with FU and lower dose of TPZ. There were no significant variations between FU and FU+TPZ groups in respect to triglycerides, glucose and proteins levels.

DISCUSSION

According to the last decade's scientific reports both *in vivo* and *in vitro* trials have shown better treatment evidence when tirapazamine was administered in combination with other chemotherapeutic agents than when administered as a sole agent. In our study we presented the results showing the TPZ influence on DOX, CP and 5-FU effects in myocytes. In our experiment, oxidative stress and chosen metabolic parameters were evaluated in skeletal muscle samples.

Doxorubicin is one of the most effective and known chemotherapeutic agents used in the anticancer therapy. However, dose-related cumulation progressive myocardial damages that may lead to lethal congestive heart failure cause that the drug is of limited value in treating cancer. So far as we noticed, much less attention is paid to skeletal muscle damage, which is probably due to extremely deferent physiological meaning of these organs. Skeletal muscles are important because patients are losing their body weight during chemotherapy according to the clinical and experimental observation. The mechanisms of DOX cardiotoxicity are well recognized as follows: disorders of Ca^{++} and Fe^{++} equilibrium [6, 9], increase in histamine and catecholamine concentrations and effects caused by both amines [3]. It is generally accepted that the mechanism of doxorubicin-induced toxic impairment of cardiomyocytes is dependent on oxidative stress which is connected with reactive oxygen species (ROS) formation [for review, see 5]. Besides the cell membranes, damage caused by lipid peroxidation in many tissue including the heart is antracycline related [11]. Concomitantly, metabolism of energetic cells disorder is observed, probably as a results of mitochondrial destruction, failure of oxidative phosphorylation and finally decrease in ATP concentration [6]. It seems highly probable that a similar type of mechanisms might happen in skeletal muscle cells. Both apoptosis and necrosis connected with myocytes death might be responsible for the total weight loss of skeletal muscles. That is consistent with what clinical observations of losing body weight by the patients treated with antracyclines stated.

Surprisingly, in our study the concentration of lipids peroxidation products (MDA+4HNE) in skeletal muscle was 5-fold lower in DOX+TPZ group rather than DOX only treated rats. It seems that TPZ restricted lipids oxidative damage observed after DOX administration, which is against our expectation. Both DOX and TPZ are reduced by NADPH dependent enzymes. Under normoxic or hyperoxic conditions, TPZ radicals are similarly as DOX radicals back-oxidised to the parent molecule with the concomitant formation of superoxide radical ($O_2^{\bullet -}$), Seemingly, it is a paradox that NADPH is simultaneously a source of oxidative stress and a main compound involved in antioxidative defence. This nucleotide is used in reduction of oxidised glutathione (GSSG) to the reduced form (GSH) – the main cellular red-ox buffer. When NADPH is used to reduce DOX and TPZ, the common pool of NADPH might be restricted to antioxidative defence. The synergistic

and additive effect of TPZ and DOX is expected in oxidative stress. Thus, we think that the rational explanation of this phenomena is the competition between DOX and TPZ for enzymatic activation. In that case, the NADPH-dependent reduction process is slower than in the presence of DOX only. Despite TPZ changing red-ox status, as observed after DOX treatment, there are no significant variances in triglycerides, glucose and proteins levels, so we can conclude that in our experimental conditions, changes in red-ox status do not significantly influence metabolic transformation of lipids, hydrocarbons and protein in skeletal muscle.

CP may also be involved in red-ox status change by lowering GSH concentration in the normal cell [10]. However the mechanism is different compared to TPZ than the enhancement of red-ox disorders, when CP is administered with TPZ could be expected. Nevertheless, there were not significant differences in MDA+4HNE concentrations between CP and CP+TPZ groups, but the levels of triglycerides and glucose were significantly lower in rats treated with CP and TPZ compared to CP only treated rats. It is well known that redo-ox equilibrium $NAD(P)H/NAD(P)^+$ plays an important role in the regulation of triglycerides and hydrocarbons metabolism [12]. These results point out a lack of linkage between metabolic disorders and red-ox status because the changes in triglycerides and hydrocarbons levels are not accompanied by oxidative stress.

In this study we demonstrated that the lower dose of TPZ administered with FU decreased MDA+4HNE level, but there were no significant differences between FU and FU+TPZ groups in respect to triglycerides glucose and proteins levels.

CONCLUSIONS

The data presented in this study support the assumption that TPZ changes the red-ox equilibrium in skeletal muscle of rats treated at the same time with DOX or FU. TPZ administered in combination with CP also caused the metabolic disorders in rats. On the other hand, the lack of relationship between oxidative stress and metabolic disorders in the tested pairs of drugs suggested that these phenomena are independent under the conducted experiment conditions. TPZ is still advancing through different *in vivo* or *in vitro* trials in single or multidrug therapy, therefore further progress in the study is expected.

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SUMMARY

Tirazapamine is a drug that has been shown to be a selective anticancer compound which was proved, in the latest studies, more efficient in concomitant treatment with other chemotherapeutic agents. In previous studies evidence suggested that the predominant mechanism DOX and TPZ cell toxicity is linked with the generation of reactive oxygen species. Moreover, under normal cell condition, CP decreased the level of reduced glutathione changing red-ox status. Then Red-ox equilibrium differences may have an impact on lipids, hydrocarbons and proteins metabolic transformations. In our study we tested the effect of TPZ in combination with DOX, CP and 5-FU on myocytes oxidative stress, triglycerides, glucose and proteins levels. The rats were treated (i.p.) with two doses (5 i 10 mg/kg b.w.) of tirapazamine, 2h before administration of 1.8 mg/kg b.w. of doxorubicin, 2 mg/kg b.w of cisplatin or 10 mg/kg b.w. of 5-fluorouracil. All tested drugs were administered once a week over a period of 6 weeks and a week after the last dose was given, and the obtained skeletal muscle samples were taken to be tasted. The data presented in this study support the assumption that TPZ changed the red-ox equilibrium in skeletal muscle of rats treated at the same time with DOX or FU. TPZ also caused metabolic disorders in rats treated simultaneously with CP. On the other hand, the lack of relationship between oxidative stress and metabolic disorders in the tested pairs of drugs suggested that these phenomena are independent under experimental conditions.

STRESZCZENIE

Na podstawie wyników badań ostatniej dekady można oczekiwać korzystnych efektów terapii przeciwnowotworowej, gdy klasyczne chemioterapeutyki będą podawane z tirapazaminą – związkiem o dużej cytotoksyczności w komórkach z hipoksją. Wiele dowodów wskazuje na wolnorodnikowy mechanizm działania cytotoksycznego tirapazaminy (TPZ) i doksorubicyny (DOX). Ponadto cisplatyna (CP) w komórkach prawidłowych zaburza równowagę red-oks poprzez zmniejszenie stężenia glutationu zredukowanego. Zmiany w stanie red-oks mogą wpływać na

przemiany metaboliczne lipidów węglowodanów i białek. Celem badań była ocena wykładników metabolizmu komórkowego i stresu komórkowego w mięśniu szkieletowym szczurów otrzymujących TPZ z DOX, CP lub 5-florouracyl (FU). Szczurom podawano TPZ w dwóch dawkach (5 i 10 mg/kg m.c.) dwie godziny przed podaniem DOX 1,8 mg/kg m.c., 2 mg/kg m.c CP lub 10 mg/kg m.c. FU. Badane związki podawano sześciokrotnie w odstępach tygodniowych. Mięśnie szkieletowe do badań pobierano tydzień po ostatnim podaniu związków. Badania wykazały, że TP zaburza równowagę red-oks w mięśniach szkieletowych szczurów otrzymujących DOX lub FU. TPZ wywołuje także zaburzenia metabolicznie w badanej tkance szczurów przyjmujących równocześnie CP. Wykazano ponadto, że zaburzenia metaboliczne w przeprowadzonym doświadczeniu są niepowiązane ze stresem oksydacyjny.