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The evaluation of rat peripheral blood morphology after coadministration of doxorubicin and tirapazamine

Ocena rozmazu krwi u szczurów otrzymujących doksorubicynę z tirapazaminą

INTRODUCTION

The results of conducted studies have shown that some of the cancer cells are typically characterized by hypoxia compared to healthy cells [1, 8, 9, 22]. These cells remain more resistant to radiotherapy and most common anticancer drugs [2, 15, 6]. Over a recent 10 years investigations regarding compounds called hypoxic-cell-selective cytotoxins have been intensified [21]. Some chemotherapeutic drug-candidates, e.g. tirapazamine, demonstrate a considerable higher cytotoxic effect against hypoxic tumor cells than normoxic cells studied under the same conditions [18, 25]. Considering this, the next step of study was to investigate their effectiveness against some cancer cell lines. Afterwards, benefits from multiple treatments with classical anticancer drugs, for instance: cisplatinum (CP), 5-fluorouracilum (5FU) and doxorubicin (DOX) together with tirazapmine (TPZ) were initiated and still continued [3]. Over the last decade treatment regimen based on CP given in combination with TPZ has been carried out in phase I and phase II clinical trials [17]. Among the above mentioned classical chemotherapeutics, DOX is the only one with similar mechanism of action a TPZ. Both DOX and TPZ are transformed by NADPH linked enzyme to toxic radical forms. However TPZ's radical is stable under hypoxic condition and induces single- and double-strength breaks tumor's cell DNA to stop cells growing and divisions [4]. Under normoxic condition free radicals of both compounds are able to transfer previously obtained electron to O_2. As a result, produced superoxide and at the same time DOX and TPZ radicals are transformed to the parent's compound [16]. These reaction circles might repeat constantly. As a consequence, superoxide production may be transformed to hydroxyl radical during Haber-Weiss and Fenton reactions. Many other reactive oxygen species might appear (e.g H_2O_2 and $ONOO^-$) and react with DNA and proteins. Considering various DOX acute side effects appearing while treatment,

changes in blood morphology seem to be the most serious [13]. However, there is no data available regarding TPZ influence on the blood morphology parameters. In this study we evaluated peripheral blood automatic smears in rats repeatedly treated with both DOX and TPZ.

MATERIAL AND METHOD

The experiment was approved by Local Bioethical Commission of Medical University in Lublin. Male Wistar rats strain having a range in body weight of 160 to 195 were purchased from Breeding Rats Brwinów/Warsaw. The rats were maintained in stable life conditions at 22˚C with a 12-h light/dark cycle and given standardized granulated fodder LSM. The experiment was carried out at the Central Animals Unit of Medical University in Lublin under supervision of Veterinary Inspectorate in Pulawy. The rats were treated (*i.p.*) with tirapazamine (3-amino-1,2,4-benzotriazine 1,4-dioxide 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. M.B.H., Austria). Both tested drugs were administered once a week over a period of 6 weeks, and a week after the last dose the blood was taken for haematological study. The animals were bled under terminal anesthesia with phenobarbital – 60 mg/kg m.c. (*Morbital; Biowet Pulawy).* Blood for biochemical and haematological evaluation was taken from the left heart ventricule into $EDTAK_3$ and clot activator containing tubes (VACUETTE®). Blood morphology test was performed using *Cell-Dyn 1700 (Abbott, USA).*

The counting of blood cells was based on the volumetric impedance method. Direct measurement hemoglobin (HGB; g/dl), white blood cells (WBC), lymphocytes, monocytes and granulocytes count, red blood cells (RBC), mean corpuscular volume (MCV; fl), and also automatic calculation of hematocrit (HCT; g/dl), mean corpuscular hemoglobin (MCH; pg), MCH concentration (MCHC; g/dl), RBC distribution width (RDW; %) were assayed. Also, platelets parameters such as platelets count (PLT), plateletcrit (PCT), and platelet distribution width (PDW) and mean platelet volume (MPV) were examined. The means of the measured values of corpuscular hemoglobin – MCH and MCH concentration – MCHC were automatically calculated from RBC, HCT and HGB values according to following formulas MCH = (HGB/RBC) x 10; MCHC = (HGB/HCT) x 100.

STATISTICA 5.0 was used to analysed the data. The statistical differences between the control group and the assessed group were compared using a U Mann-Whitney's test or t-Student's test. ANOVA was used to compare the means between particular tested groups. The post-hoc test NIR was used to verify null hypothesis according to the thesis that evaluated parameters in rats receiving DOX are influenced by TPZ. All data are expressed as mean ± SD. The value of $p<0.05$ was considered statistically significant.

RESULTS

The major finding of the presented study is that antitumor agent TPZ significantly changed the relative amount of lymphocytes in rats where DOX was administered (tab. 1). There were no changes versus control in all white blood parameters tested in rats treated with a higher dose of TPZ in spite of a lower dose of TPZ declined number of granulocytes. The only interaction between DOX and TPZ among red cells blood parameters was demonstrated exclusively referring to the MCV (tab. 2). Significant increment of RDW in every tested group except 10TPZ comparing to the control was observed. Platelets parameter changes were found exclusively in the group receiving DOX combined with lower dose of TPZ (tab. 3).

Table 1. White blood cells parameters (mean ± S.D.); p – statistical significance vs. control,
statistical significance ($p \leq 0.05$) vs. group of DOX

Group/parameter	Control N=7	DOX N=6	DOX+5TPZ N=6	DOX+10TPZ N=6	5TPZ N=7	10TPZ N=5
WBC (10^9/l)	7.07±1.291	6.40±2.883 p=0.5883	5.29±2.624 p=0.1396	6.77±1.029 p=0.6512	6.87±2.584 p=0.8577	6.42±0.955 p=0.361895
LYM (10^9/l)	4.87±1.083	3.43±1.019 **p=0.0321**	2.34±1.683 **p=0.0073**	4.12±0.801 p=0.1864	5.57±2.087 p=0.4461	4.37±1.186 p=0,4607
MON (10^9/l)	0.54±0.162	1.03±0.489 **p=0.0383**	1.37±1.058 p=0.0633	0.98±0.384 **p=0.0151**	0.54±0.190 p=1.0000	0.63±0.194 p=0.3961
GRAN (10^9/l)	1.66±0.264	1.97±1.697 p=0.3531	1.80±0.391 p=0.5707	1.78±0.383 p=0.5230	0.76±0.346 **p=0.0032**	1.33±0.150 p=0.0587
LYM (%)	69.24±4.583	58.07±11.620 **p=0.0455**	42.32±15.371# **p=0.0042**	61.10±5.542 **p=0.03212**	81.70±2.040 **p=0.0017**	67.96±11.039 p=0.7853
MON (%)	8.44±1.625	16.72±1.040 p=**0.0000**	25.40±10.512 p=**0.0027**	15.07±6.357 p=**0.0034**	8.60±0.968 p=0.4062	10.72±4.195 p=0.2148
GRAN (%)	22.31±3.734	25.17±12.023 p=0.7750	25.27±5.437 p=0.4496	24.76±4.045 p=0.3047	9.70±1.881 **p=0.0017**	18.35±2.259 p=0.0888

Table 2. Red blood cells parameters (mean ± S.D.); p – statistical significance vs. control, # statistical significance ($p \leq 0.05$) vs. DOX group

Group/parameter	Control N=7	DOX N=6	DOX+5TPZ N=6	DOX+10TPZ N=6	5TPZ N=7	10TPZ N=5
RBC (10^{12}/l)	7.72±0.89	7.01±0.37 **p=0.0321**	6.89±1.19 p=0.0864	7.33±0.37 p=0.0864	8.14±0.31 p=0.2774	8.11±0.45 p=0.2556
HGB (g/dl)	14.10±1.43	12.85±0.56 p=0.0705	16.55±10.54 p=0.5522	13.55±0.80 p=0.4223	14.56±0.62 p=0.4533	14.44±0.46 p=0.6233
HCT (ratio)	40.53±4.97	36.90±1.84 p=0.1203	31.15±10.90 p=0.0647	39.82±2.04 p=0.7501	44.88±1.96 p=0.2574	41.34±1.46 p=0.7335
MCV (fl)	52.57±1.512	52.67±0.516 p=0.8862	50.93±1.402# p=0.0693	54.32±1.662# p=0.0728	52.71±1.604 p=0.8666	51.14±2.459 p=0.2378
MCH (pg)	18.34±0.941	18.32±0.605 p=0.9544	18.08±1.169 p=0.6656	18.50±0.352 p=0.7079	17.90±0.566 p=0.3070	17.84±0.841 p=0.3638
MCHC (g/dl)	34.94±1.952	34.83±1.075 p=0.9050	35.45±1.908 p=0.6462	34.07±0.829 p=0.3302	33.90±0.837 p=0.2182	34.96±0.279 p=0.9850
RDW (%)	13.17±0.865	14.97±1.097 **p=0.0070**	16.85±1.763 **p=0.0004**	15.87±1.223 **p=0.0007**	14.06±0.420 **p=0.0313**	14.06±0.251 p=0.1752

Table 3. Platelets parameters (mean x ± S.D.); p – statistical significance vs. control,
statistical significance (p ≤0.05) vs. DOX group

Group/parameter	Control N=7	DOX N=6	DOX+5TPZ N=6	DOX+10TPZ N=6	5TPZ N=7	10TPZ N=5
PLT (10^9/l)	967.86±162.041	1199.83±238.536 p=0.0616	1501.17±131.893 **p=0.0001**	1170.67±413.108 p=0.2544	933.57±108.206 p=0.6498	1036.20±235.593 p=0.5624
MPV(fl)	7.69±0.780	7.17±0.625 p=0.2177	7.13±0.720 p=0.2142	7.02±0.595 p=0.1145	7.69±0.788 p=1.0000	7.77±0.547 p=0.8353
PCT (%)	0.75±0.159	0.86±0.190 p=0.2673	1.08±0.164 p=**0.0039**	0.95±0.215 p=0.0848	0.72±0.137 p=0.7253	0.77±0.141 p=0.7887
PDW (%)	6.73±2.04	7.52±0.46 p=0.8303	46.83±32.58# **p=0.0124**	10.62±6.33 p=0.2839	6.61±1.31 p=0.7982	25.28±41.57 p=1.0000

DISCUSSION

Changes in peripheral blood count are well documented as a side effect during DOX therapy [13]. However, according to our best knowledge the effect of TPZ on these changes has not been published yet. Toxicological screening is a very useful tool which can eliminate the concept of improved therapy planned with DOX and TPZ administered together. These findings raised the unanswered question about evaluation of interactions between DOX and TPZ. Our investigation is only a part of a broader project aimed to evaluate the organs toxicity in that therapy schedule.

TPZ has shown anticancer activity through its ability to selective DNA damage in the hypoxic cells which are found inside solid tumors [18, 25]. It has also undergone clinical trials as well as cisplatine and/or radiotherapy referring to the lung, cervical, head and neck cancers [17, 19]. However, up to now there has been no apparent clinical evidence indicating improvement of the classical therapy efficacy in phase III of clinical trials. TPZ is a potentially non-toxic compound that is activated via one electron reduction to a reactive-drug free radical, oxygen-sensitive [11] with cytotoxic properties causing double-strand DNA breaks and consequently chromosome aberration and then cell killing under hypoxic conditions. The one-electron enzymatic reduction is catalysed by NADPH dependent: cytochrome P450 reductase, and is thought to be responsible for *in vivo* activation of TPZ [23]. Also xanthine/xanthine oxidase has been used successfully for the activation of TPZ in a variety of *in vitro* studies [7]. Similarly, this one-electron reduction process is also a well known mechanism occurring during turn-over of DOX – DNA intercalating agent [14]. Every suppressor of mitogenic activity is able to cause damage to a high proliferating tissues, specially to a various blood cells (erythrocytes, granulocytes, platelets) each produced at a rate of approximately 1–3 million per second in a healthy adult [12]. Consequently, an early and most common side effect is myelosuppression associated with the administration of antimitotic agents. The current literature data has shown that during DOX treatment leucocytes count may drop and this is an indication of the dose reduction or therapy cessation when the level of leucocytes is below 1500. At the same time, the red blood count and platelets count may seriously diminish. The therapy should be stopped when platelets reach 30–50 x 10^9/l.

Interestingly, there are no changes in WBC, RBC and PLT count in the group of rats treated with TPZ. Based on this evidence we found that TPZ did not show myelotoxic activity. However, Holden and al. [1992] described the cytotoxic effect on bone marrow cells isolated from C3H/FeJ mice bearing the FSaIIC fibrosarcoma. Moreover, Spiegel and al. [1993] found bone marrow suppression after repeated 6 weaks' (5 times a weak) administration of TPZ. Results of another hematological study [24] demonstrated that TPZ was well tolerated while this compound was given for 5 days every 6 weeks and blood was taken for the analysis after 3-weeks recovery period. The authors stated that bone marrow was a major targeted organ. In our study, despite no changes in WBC, RBC and PLT we noticed a statistically significant increase in RDW value and a significant diminishment in granulocytes and an increase in LYM% in rats treated with TPZ only. It seems that the observed changes in WBC parameters tested are not clinically important because the obtained levels of lymphocytes and granulocytes are within the reference range for 7–14 weeks' Wistar rats [5]. Interestingly, administration of TPZ only in a lower dose results in some white blood cells parameters changes but it was not observed in rats treated with twice a higher dose.

Similarly, referring to the group treated with DOX only, we fund that DOX and a lower TPZ dose given together resulted in a several-fold increase in the PDW value and at the same time, no difference between DOX+10TPZ versus DOX was observed. Occasionally, an increase in the PDW value can be caused by platelets agglutination and erroneously interpreted as anisocitosis. However, the analytical error is excluded because no differences of MPV value were shown in DOX+5TPZ group comparing to the control and we have to eliminate this hypothesis. Platelets anisocytosis may result from insufficiency of iron as well. That seems to be a reasonable explanation because iron deficiency may also cause an increase in RDW and platelets count, which simultaneously appeared in DOX+5TPZ group. We can expect that platelets anisocytosis observed in that group may result in hemostasis disorders. Also, a stronger and significant decrease in LYM% was observed in DOX+5TPZ group versus DOX with no difference between DOX+10TPZ versus DOX. The current literature data on tirapazamine leaves unanswered questions about mechanisms of biological activity of TPZ and hence we cannot explain this phenomenon.

CONCLUSIONS

Collectively, these findings suggest interactions between TPZ and DOX in relation to some red cells, white cells and platelets parameters, but clinical relevance of this phenomenon seems to be acceptable during chemotherapy. However, future studies are needed to assess the importance of anisocytosis as a result of DOX-TPZ interaction.

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SUMMARY

Some cancer cells are typically characterized by hypoxia which is more resistant to radiotherapy and most common anticancer drugs. These specific phenomena encouraged researchers to the investigate new hypoxic-cell-selective cytotoxins. Tirapazamine (TPZ) is a representative of the drug-candidates group which demonstrates a considerable higher cytotoxic effect against hypoxic

and then normoxic tumor cells studied under the same conditions. Taking into account that solid tumor does not only consist of hypoxic but also normoxic cells, a rational therapy should combine administration of classical anticancer therapy and TPZ. Results of some studies conducted under laboratory conditions indicated some benefits of such a schedule. Because doxorubicin (DOX), a widely used chemotherapeutic drug, has a similar mechanism of action as TPZ, we tested the hypothesis assuming interaction of both compounds in rats referring to blood toxicity. In this study we evaluated peripheral blood automatic smear in rats repeatedly treated with both DOX and TPZ. The major findings are that TPZ may statistically significantly change the relative amount of lymphocytes, MPV MCV and RDW in rats treated with DOX. Collectively, these findings suggest interactions between TPZ and DOX in relation to some red cells, white cells and platelets parameters, but the clinical risk of this phenomenon seems to be acceptable during chemotherapy. However, future studies are needed to assess the importance of anisocytosis as a result DOX-TPZ interaction.

Keywords: doxorubicin, tirapazamine, blood toxicity

STRESZCZENIE

Komórki wielu nowotworów charakteryzuje niska prężność tlenu, której towarzyszy oporność na radioterapię i klasyczną chemioterapię. Takie specyficzne zjawisko zachęciło do badań nad związkami grupy cytotoksyn hipoksyjnych. Przedstawicielem tej grupy związków jest tirapazamina (TPZ), wykazująca znacznie wyższą toksyczność w stosunku do komórek nowotworowych w warunkach hipoksji niż w normoksji. Biorąc pod uwagę, że w guzach litych oprócz komórek z normoksją obserwuje się także strefy z hipoksją, wydaje się uzasadnione, aby podczas klasycznej terapii przeciwnowotworowej stosować również związki o specyficznym powinowactwie do komórek z niską prężnością tlenu. Wyniki dotychczasowych badań przeprowadzonych w warunkach laboratoryjnych wskazują na korzyści z takiego postępowania. Ze względu na to, że doksorubicyna – klasyczny lek przeciwnowotworowy – wykazuje podobny mechanizm działania jak TPZ, w przeprowadzonych badaniach testowano hipotezę, zgodnie z którą podawanie jednoczesne DOX i TPZ wywołuje toksyczną interakcji w odniesieniu do komórek krwi. W badaniu oceniono automatyczny rozmaz krwi u szczurów otrzymujących wielokrotną dawkę DOX z TPZ. Stwierdzono, że TPZ może w statystycznie istotny sposób zmieniać względną liczbę limfocytów, MPV, MCV i RDW u szczurów otrzymujących DOX. Uzyskane wyniki sugerują, że TPZ wywołuje interakcje z DOX w stosunku do niektórych parametrów panelu białokrwinkowego, czerwonokrwinkowego i płytek krwi, ale kliniczne ryzyko tego zjawiska wydaje się możliwe do zaakceptowania podczas chemioterapii. Bardziej szczegółowego wyjaśnienia wymaga jednak znaczenie anizocytozy obserwowanej jako wynik interakcji DOX z TPZ.

Słowa kluczowe: doksorubicyna, tirapazamina, toksyczna interakcja