

Examination of the Side Effects and Consequences in Cyclophosphamide Monotherapy with DSC in Animal Models

Ph.D. Thesis Summary

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Introduction

In the 21st century, throughout developed countries, cardiovascular diseases are followed by malignant tumors as the second leading cause of death. According to the data of the Central Statistical office, while cardiovascular diseases as the cause of death show a minimal declining trend, on the other hand, in contrast, the second most frequent cause of death, is the incidence of malignant tumors, of which, today, illustrates a slight upward trend.

Due to the above tendencies, an increasing emphasis is placed on the cure and treatment of tumorous diseases. In the overwhelming majority of malignant spatial processes, the principal objective is the first in consideration of the total excision of the lesion. Unfortunately, in many cases, it is not possible, thus, irradiation, radiotherapy and chemotherapy play a key role depending on the characteristics of the tumor. Strikingly, both options possess risk, including serious side effects.

Determining the proper dose during chemotherapy is mostly based on an improved method or in the consideration of developing international recommendations and standards. However, with respect to individualized, patient-tailored dosage, the specified treatment is not possible. In considering the pharmacokinetics of most medications and chemotherapeutic agents, which are influenced partially on a genetic basis or partially upon the current general wellbeing of the patient, the same dose may have a vastly different effect on over a wide spectrum of patients.

Cyclophosphamide was chosen for our study as similar to oncotherapy; it plays a prominent role throughout other disease groups, mostly in immunology, and is widely used as a part of prolonged therapy. It is also applied in dermatology and pulmonology, although with significantly less impact. Due to this, the characteristics of the potential side effects play a major role, and hopefully, clinical fields of research may soon benefit from the result of our experiments.

The importance of cyclophosphamide is suitably demonstrated, as it is included in the WHO list of essential medicines (2015 April).

Objectives

The aim of the dissertation is to assess DSC (Differential Scanning Calorimetry) as a potential clinical diagnostic tool *in vitro*, based on our experiments. We intend to prove the suitability of the method in predicting the complications and in accurately estimating the expected side effects of the systemic therapy when considering the administration of cyclophosphamide.

In our animal models, when judging the effects of the medication following the administration of cyclophosphamide therapy (intraperitoneally administered human equivalent dose), we separately examined nerve-muscle complex, heart muscle, blood plasma and RBC (red blood cells) suspension. We intend to judge the following:

- alterations due to changes on nerve-muscle complex towards neuropathy and motility dysfunction
- alterations developing in the heart muscle which may have a role in evoking cardiomyopathy
- detectable alterations on the formidable elements of blood, RBC and blood plasma, which may provide an indirect method in monitoring the drug level and to estimate the functional alterations developing during long-term treatment
- predictable value of dose-dependent changes, based on our results, concerning possible consequences.

Our Experiments and Results

Adult guinea pigs (*Cavia porcellus*) were injected intraperitoneally with cyclophosphamide. Intraperitoneal drug administration in pets is a widely applied method which can be considered equivalent to human intravenous use. Research permission was requested, approval number: BA02/2000-4/2012.

Onset of examination

The idea of using the DSC method was inspired by the intention to solve a specific forensic case. To determine the answer, guinea pigs were injected intraperitoneally with the dose of cyclophosphamide, which is comparable to the human protocols (5 mg/kg) 2-5 times consecutively, including several days of pause, in accordance to the human protocol. Non-injected guinea pigs were used as control agents.

Guinea pigs were terminated in chambers filled with narcotic ether, and then sciatic nerves and gastrocnemius muscles were removed from the lower limbs. Two centimeter-sized sciatic nerve samples and 0.5 x 0.5 x 2cm muscle samples from the same anatomical site were removed. The samples were kept in sterile saline solution at 4°C until the calorimetric examination phase, typically, within 12 hours after acquiring the samples.

DSC measurements

The prepared samples were next washed three times in a normal saline to remove foreign objects and residual tissue prior to the calorimetric examinations.

The thermal analysis was made using a SETARAM Micro DSC-II calorimeter, between 0 and 100°C, with a heating rate of 0.3 K/min. Conventional Hastelloy batch vessels (V=1 mL) were used for the experiment to determine denaturation with 950 µL sample volume (sample + buffer) in average. Sample masses were between 250-400 mg. Isotonic saline solution was used as a reference. The reference and sample vessels were equilibrated with a precision of ± 0.1 mg, assuring there was no need to correct between the vessels' heat capacity. With the help of a two-point setting, SETARAM peak integration calorimetric enthalpy was calculated from the area beneath the heat absorption curve, and then, the results (max. denaturation temperatures of different samples TM, its numbering refers to a tool which can be divided into several thermic domains), and the calorimetry enthalpy (ΔH) were compared.

The thermal denaturation of the healthy, 'control' (Fig.1) gastrocnemius muscles is similar to the thermal absorption curves of the actomyosin solutions and rabbit psoas muscle fibers. Based on this, the first denaturation peak is in connection with the myosin

head, the second peak refers to the tail domain (myosin rod) while the third peak relates to the thermal denaturation of actin filaments. The thermal denaturation of samples of cytotoxic-treated animals, based on the DSC-curves, demonstrated dose-dependent changes (Fig.1).

Based on the obtained data, the myosin head (T_{m1}) appears to be the most sensitive to the therapeutic dosage. The first dose can be interpreted as a shock effect due to the extremely high T_{m1} , while additional doses resulted in nearly the same degree changes. The tail domain (myosin rod) (T_{m2} values) and the actin (T_{m3} values), following the second and fourth dose of cytostatic treatment, offered a significantly different result compared to the control. The structural changes are also well monitored by the calorimetric enthalpies; it also proved to be dose-dependent. Due to the limited number of measurements (only one treatment procedure was performed together with the control in a group of five individuals) statistically proved correlation was not traceable, although the tendency is well demonstrated. Therefore, chemotherapy treatment may have damaging effects upon the motility system.

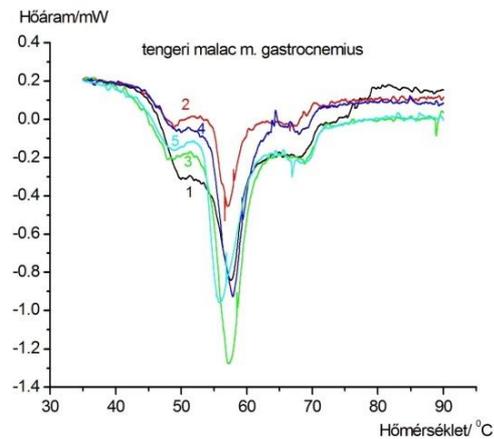


Fig. 1.: Thermal denaturation curves of guinea pig m. gastrocnemius treated with different doses of cyclophosphamide (1=control, 2-5 the dose increases)

In reference to the functionality of the motility system (muscle), it does depend not only on its structural characteristics, but on its innervation, too. Additionally, measurements were performed on the ischiadic nerve, which is responsible for muscle function.

On the basis of Fig. 2, the denaturation heat flow curve of the nerve is notably dose-dependent.

On the basis of thermal data, most of the administered chemotherapy doses caused structural changes (either by T_m or ΔH).

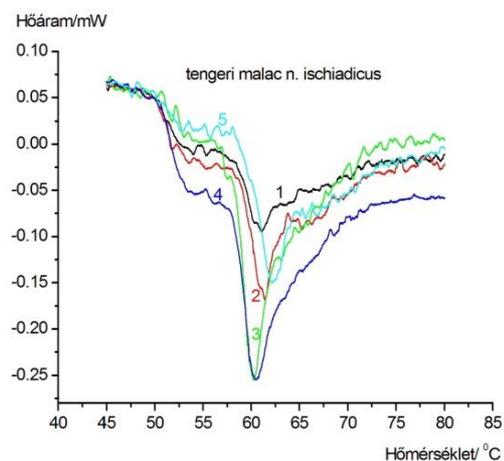


Fig 1.: Thermal denaturation curves of guinea pig ischiadic nerve treated with different doses of cyclophosphamide (1=control, 2-5 the dose increases)

In summary, the examined nerves demonstrate increased susceptibility to the chemotherapeutic drug when compared with the innervated target muscle.

Discussion

Polyneuropathy can be caused by numerous endogen and exogen factors. In the case of malignant diseases, as the etiology of neuropathy, paraneoplastic symptoms arise first in many cases, although it may likely be proven, in which mostly the applied chemotherapeutic drug administration may be responsible, however, their direct neurotoxic effect is only partially substantiated. The study was inspired by the forensic investigation of an ovarian cancer (adenocarcinoma) survivor, a young female patient, in whose case the development of polyneuropathy could have been caused by cyclophosphamide therapy. Animal experiments proved neuro- and myotoxic effects of cyclophosphamide, while guinea pigs were administered the dosage of cyclophosphamide, comparable with human therapeutic protocols. Calorimetry is suitable in identifying different characteristics and levels of muscle damage, based on data published throughout international literature. However, calorimetry has not been used in the examination of peripheral nerves.

Conclusion

As the study attempted to provide an answer to a specific clinical case, only a low number of measurements were performed. In this way, and largely based on this criteria, a significant result cannot yet be entirely and effectively presented. Although, it can be stated that, both peripheral nerve and muscle structural changes developed due to cyclophosphamide therapy, which are measurable and verifiable when implementing calorimetry. Additionally, it can also be stated, that the rate and extent of changes are indeed dependent upon the dose of cyclophosphamide. These results provided a stable base for planning and performing further examinations by our lab.

Implementation of new investigations

Based on the promising results of the initiative investigation, including a low number of specimens, we intended to continue our investigation series once number of specimens was increased up to or more than ten times and several types of dosage schemes were used. The difference between the two extreme mass values of the guinea pigs used during the experiments is almost five fold (230 g and 1200 g) and, when considering the majority of the specimens, there was also an immense spread. Identically to the initial investigation, each guinea pig was administered a unique absolute dose of cyclophosphamide intraperitoneally, separately calculated when considering the body mass (5 mg/body mass kg). DSC measurements were identical to the initial investigation in circumstances and parameters.

In our experiment, in 11 different groups including 5-5 animals (n=55, n=5/group) and 11 different dosing schemes were set up in which drug administration was repeated 1-6 times, partly on consecutive days, partly including several days of interval. It is to be noted that, 2 guinea pigs from the group receiving 6 treatments succumbed prior to the end of the experiment, following the 5th treatment. The reason is yet unclear. To evaluate our results, a

control group was used, in which the examination of non-injected guinea pigs (n=5) was performed under the same conditions using the same method.

Guinea pigs were terminated in chambers filled with narcotic ether, and then, samples were taken from different sites:

- gastrocnemius muscles of size 0.5 x 0.5 x 2 and 2 cm sciatic nerve samples were removed and prepared from the specimens from both sides of the torso, and from the same anatomical site
- 0.5 x 0.5 x 0.5 cm sample of left ventricle cardiac muscle was removed
- peripheral blood samples were collected into the vacutainer tubes containing EDTA (1.5 mg/ml blood) then centrifuged at 1600g for 15 min at 4°C to separate plasma fraction from cell components (RBC).

The nerve and muscle samples were kept in a sterile saline solution (max. 12 hours) until the onset of the examinations.

Investigation of nerve-muscle complex

The extension of the initial examination was planned to support and improve the achieved results with the increase of dosage scheme numbers.

The specimens were divided into 7 larger groups on the basis of the number of treatments (1-6), in addition to the control group. Due to the difference in tissue characteristics, although they form a functional unit, gastrocnemius muscles and sciatic nerves were examined separately. In every specimen, pig nerve-muscle complexes were prepared from both sides, thus double measurements were made in every case, independently representing both right and left side samples.

Fig.3 demonstrates dose-dependent effects of cyclophosphamide treatment on peripheral nerves.

The maximum decrease of the heat flow is accompanied with the shift in the denaturation temperature into a smaller range, dependent upon the dosage. An unexpected alteration was witnessed and was well demonstrated in the values, specifically, the left side nerves were more affected by the treatment. This phenomenon cannot yet be clearly explained. It should be noted, however, in every case, the drug was administered into

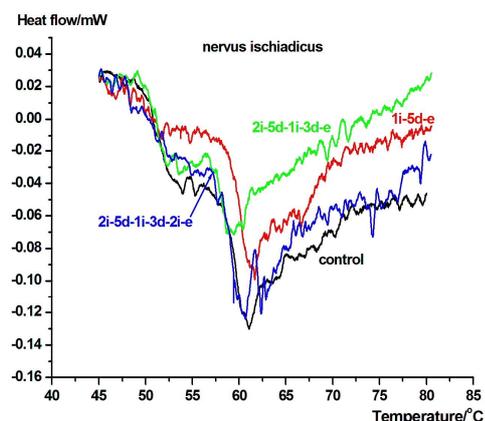


Fig. 2.: Thermal denaturation curves of guinea pig ischiadic nerve treated with different doses of cyclophosphamide (i=injection, d=days passed)

the left side of the abdomen, but this in itself cannot account for the difference.

In case of right-sided nerves, the thermal domain characterized by T_{m3} and the total calorimetric enthalpy can show the dose dependence of the treatment, while in T_{m1} and T_{m2} changes, we could only observe a very mild tendency (only during 1-2 injections). Largely, it is dependent upon the number of injections and the time intervals between the injections, which proves significant (decreases). Thereafter, only after administering six injections (the maximum) could we observe significant dose dependence in T_{m1} . On the other hand, the same data in case of left sided nerves show that the strong dose dependence of the thermal stability of thermally separable structural domains is followed by T_{m1} and T_{m3} , after only to two injections. Compared with the control examinations, the dose dependence of calorimetric enthalpy is clearly practical in every case, however, the effect of the 2nd and 3rd injections increases, compared to the former cases.

Within the Department of Biophysics, Medical School, PTE, investigation of striated muscles has already been performed with DSC, originating from different aspects, and so, we have a significant advantageous experience in the evaluation of measurement data of the gastrocnemius muscle.

The heat denaturation curves of the removed gastrocnemius muscles can be seen on Fig. 4:

The curve of the denaturation heat stream can be divided into 3 compartmentalized sections. Based on previous investigations, the middle section of duration serves as myosin rod's, the lower denaturation peak equals myosin head's, while the highest equals the actin and actin-myosin complex thermal denaturation. Heat denaturation curves accurately demonstrate the dose dependent changes of cyclophosphamide treatment. Thermal parameters, similar to sciatic nerves, show the dominance of damage in the left side.

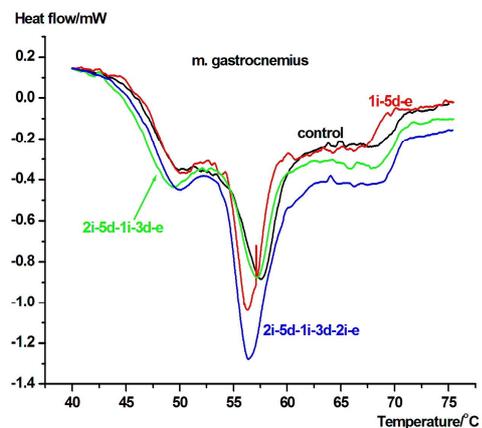


Fig. 3.: Thermal denaturation curves of guinea pig m. gastrocnemius treated with different doses of cyclophosphamide (i=injection, d=days passed)

Structural changes, due to chemotherapy, are demonstrated by the modifications in tendency of denaturation thermal points (T_m) and calorimetric enthalpies (ΔH_c). Based on this, during every treatment, mainly actin filaments' involvement was witnessed in the right muscle with a similar change in enthalpy. In the case of the left muscles, the myosin head is also involved, in addition to a higher significant enthalpy dose-dependence. All of this physiologically causes the deterioration of actin-myosin complex, which later results in weaker movement skills.

Discussion

Cyclophosphamide has a wide spectrum of relatively severe side effects; among them is the peripheral nerve and muscle damage including the possibility of long term consequences, such as polyneuropathy. Although, the neurotoxic effect of chemotherapeutics is partially proven, its possibility has already been suggested in the case of cyclophosphamide. DSC investigation methods confirmed its suitability to detect muscle damage, while investigations have been performed in the detection of nerve damage within a smaller number for experimental use.

Based upon our results, we can state, that changes in the thermal parameters clearly prove the damaging effects of cyclophosphamide in peripheral nerves and to a lesser extent, in the case of striated muscles. The deviation can mainly be explained in considering the difference of tissue sensitivity, however, both lead to functional damage, and their effect may accumulate. The extent of the changes correlates to the applied dosage.

It is a point of interest, in our investigation, in which we noticed a clear side-difference, which proved a more intense involvement of the nerves and muscles in the left side. Its background is not yet clarified, however, the aspect is worth investigating, since later, in the treated patient population, a similar predominance can be substantiated, and then it is worth consideration during the medical check-up and in the evaluation of control results.

Conclusion

Based on the results of our initial investigation, in which the animal experiment in a larger number (n=55), with the help of differential scanning calorimetry (DSC), the significant detection of thermochemical changes clearly confirmed the harmful effects of dose-dependent cyclophosphamide in the peripheral nerves and muscles. Based on these findings, the harmful effects of cyclophosphamide also surface throughout other fields of research, of which, now require additional investigation. The side-difference also raises further questions.

Investigation of heart muscle

During the strategic planning of our investigation, the examination of the heart muscle was established on the fact in which one of the most severe, often fatal side effects of cyclophosphamide and several other cytostatics, is cardiac toxic effect, or cardiotoxicity. The pathophysiology of the process is relatively well known, however, the triggering dose and the predictive signs are not yet fully clarified, and thus, in considering the severity of the possible consequences, its examination is deemed worthy.

Samples were removed from the left ventricular of the examined specimens, identical to the nerve/muscle experimental group, as it is exposed the highest strain.

The dose-dependent effect of cyclophosphamide, the shift of denaturation temperatures and the decrease of enthalpy can be well observed in the heat denaturation curve of the left ventricular (Fig. 5.)

To effectively analyze our results, we used the experience and consequences gained during the previous examinations of the skeletal muscle. Much like the case of striated muscle, the denaturation curve of the heart muscle can be divided into three sections. According to our experiences, the melting point is at or about 58°C (in control the middle peak), which is suitable for the denaturation of the myosin rod, the lower shift for the myosin head while the highest peak over 62°C, is suitable for the denaturation of the actin resp. actin-myosin complex. The modification of all these, concerning the amount of administered cyclophosphamide can be characterized with the course of the curves (form) and with the shifts of max. heat flows.

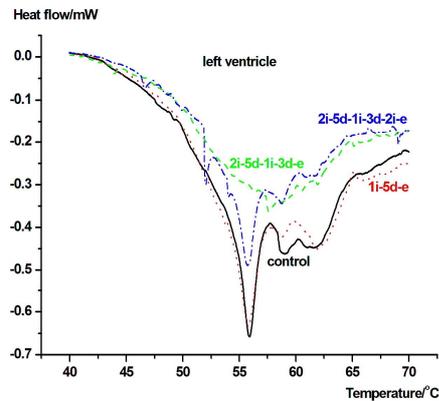


Fig. 4.: Thermal denaturation curves of guinea pig left ventricle treated with different doses of cyclophosphamide (i=injection, d=days passed)

The measured values demonstrate how that 1-2 cyclophosphamide treatments influence the T_{m1} , T_{m3} and the calorimetric enthalpy as well, meaning, it can be concluded, in which the myosin head and the actin are more sensitive to treatment. Although, the calorimetric enthalpy is more dependent upon the dosage and the time elapsed between treatments. In the event of a greater number of treatments, during 3 or more, the change of T_{m2} , T_{m3} and calorimetric enthalpy is able to monitor the expressed effect, in which, mostly, the myosin rod and the actin are involved. Regarding the entirety of the process, we state, that primarily the myosin heads and the actin filament suffered the change (T_{m1} , T_{m3}), which generated a decreased actomyosin complex function, eventually leading to a decreased functionality of the heart pump.

Discussion

Cyclophosphamide induced myocardial damage and hemorrhagic myocarditis is known throughout published literature, however, their mechanism is only partially clarified and the standardization of guiding dosage limits has not yet been proved successfully. Considering the wide usage of the medication and the possibly fatal outcome of the complications, the more precise recognition of the process plays an important role. As of yet, the effect of cyclophosphamide upon the heart muscle has not been investigated with DSC. Our research team performed the investigations including oncological indication upon guinea pigs under experimental conditions mindful of the dose and protocol of bodyweight/kg.

In consideration of the experience of our previous examinations and based on our results, we state, that in multiple treatments regarding cyclophosphamide, it characteristically first damages the myosin head and the actin, then, by increasing the dosage, the complete actomyosin complex suffers damage, which likely plays an important role in the developmental clinical picture.

It can also be stated; a single cyclophosphamide treatment did not cause significant heart muscle damage, which advances the belief in which, it is clearly supportive as evident, the treatment is indeed, dose-dependent, despite the fact in which a cumulative feature has not yet been detected in former examinations.

Conclusion

Hopefully, with our examination and results, we have made further steps towards the comprehension and clarification of cyclophosphamide induced cardiomyopathy from the pathophysiological aspect. Additionally, we succeeded in offering a new direction to effectively characterize the estimated dose-limit.

Examination of plasma and red blood cells

Examination of the blood was considered to be justified, since it properly reflects the effects on the body, but it is even more important, since it is one of the biological samples which can be tested nearly always in the easiest and best way throughout the daily clinical routine, and it can provide relevant information on the patient's condition. Expanding literature is available in the examination of blood with DSC, in the case of several different pathological deviations. So long as we can find significant alterations, preeminently, if they correlate with the applied dosage, we possess the possibility of a new method, which is able to monitor, possibly predict the processes and/or their severity in other areas of the body. If and once that succeeds, there is a chance to prevent or moderate the drug-induced side effects and severe consequences which sometimes accompany prescription medication.

Blood samples were taken from the same animals, as formerly described, from the nerve, muscle and heart muscle samples, and in this way, the dosage scheme and the grouping are identical.

Effects of cyclophosphamide on the plasma are demonstrated in heat denaturation curves depending on the dosage (Fig.6.). To make it clear, in addition to the control, only 3 different (after 1, 3 and 5 treatments) denaturation curves are represented in which the tendency of the entire process can be seen. In the case of T_{m1} (which, according to the literature stands for the denaturation of fibrinogen), and T_{m3} (denaturation of globulins) except for the first treatment, the denaturation heat points moved to the lower range, while the maximum heat flow showed a decreasing tendency to every treatment.

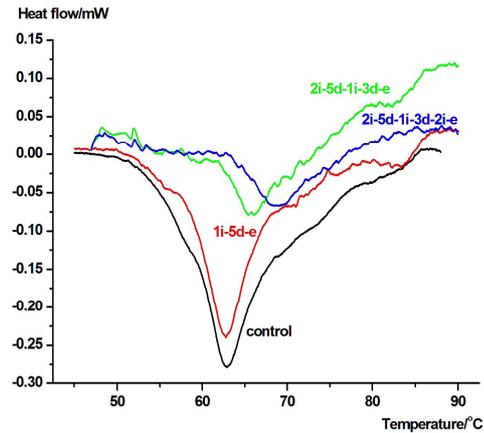


Fig. 6.: Thermal denaturation curves of guinea pig blood plasma treated with different doses of cyclophosphamide (i=injection, d=days passed)

The denaturation characterized by T_{m2} , which derives from the component of the plasma albumin, is significantly higher in every case, than when compared to the control. Generally speaking, in the examined blood plasma samples, we can assume, in which throughout every case, at least three different thermal structural units are present (without deconvolution). Due to the cyclophosphamide treatment, the more strongly influenced domains can be characterized by the shift of T_{m2} and T_{m3} values. Due to the onset of cyclophosphamide therapy, following the first injection, a definite and significant increase can be seen in the denaturation maximum temperatures (T_m), which are strongly influenced by the post-treatment period and the number of the treatments. The decrease of calorimetric enthalpy becomes significant only after the second treatment. In reference to the third injection, however, a significant decrease develops in the thermal parameters of T_{m1} , T_{m3} and ΔH_c .

In the case of RBC, we can find an interesting difference, namely in the heat denaturation curve of the control group, in which a 4-step isolable denaturation develops (Fig.7.). In the expected range, 3 endothermic reactions occurred (at 69, 76 and 84 °C). These transformations shift to lower temperatures with the increase in the number of treatments, while after the fifth treatment, there is practically only one endothermic reaction throughout the system.

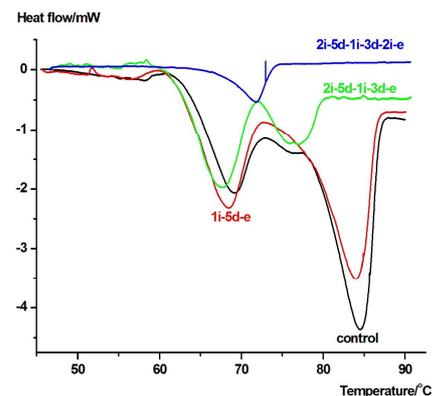


Fig. 7.: Thermal denaturation curves of guinea pig RBC treated with different doses of cyclophosphamide (i=injection, d=days passed)

In recognizing these characteristics, it may now play an important role in strategically planning medical treatment, throughout clinical practice, and requires a strict control regarding human use. In the first group (termination on the day after the injection), the reaction T_m of the two endotherms featuring higher temperatures, significantly decreased. In contrast, within the groups in which the animals underwent two treatments, only T_{m3} decreased significantly, while T_{m1} and T_{m2} values demonstrated distinct fluctuations. In the groups receiving 3 and 4 doses of cyclophosphamide treatment, all melting temperatures (except in the sixth treatment), significantly decreased. Calorimetric enthalpies showed a significant increase after the first and fourth injections while there was a decrease in the others, in which the period prior to termination plays a more obvious role.

Discussion

In our research we investigated the effects of the widely used cyclophosphamide on the blood plasma components and on RBC. Based on our results in animal models, we state, that due to the effect of cyclophosphamide treatment comparable with human therapeutic protocols, both RBC and blood plasma showed changes, detectable with DSC. The detected changes presumably, are partially associated with the concentration of the cyclophosphamide plasma, and partially with the biological effects of the agent on the plasma components (on the basis of literary data, mainly on fibrinogen, albumin and globulins) and RBC, which most likely also includes functional consequences, on the basis of biological laws. These alterations may play a role in the different side effects, in which the degree of the alteration has a predictive feature concerning its severity and course.

Upon introspection, in which relatively fast and informative determination of cyclophosphamide plasma level is rather difficult in the course of a daily routine, the use of this method and its new direction, offers new possibilities. Additionally, to predict with more accuracy, the known, unfavorable and sometimes fatal side effects of cyclophosphamide is also important, which in accordance to our research, expectedly may correlate with the degree and quality of the effect upon the blood components. To assess it more precisely, additional investigations may likely be required, mostly in light of the fact, in which the clinical use of our method (DSC) throughout other fields is currently in process and the number of publications are increasing.

Conclusion

Our investigations clearly prove the detectability of cyclophosphamide induced changes on RBC and on the elements of the plasma with the help of DSC, which offers a new approach to assess the predictability of the expected effects and undesired side effects. The process of clinical usage of DSC is promising, thus, requires further investigations in which invaluable medical findings will likely be found with the use of this testing procedure.

Evaluation of our experience series

Throughout our investigations, we endeavored to demonstrate cyclophosphamide induced effects and alterations with an already known but not yet widely distributed method, namely, in the operational use of Differential Scanning Calorimetry (DSC).

The cyclophosphamide induced effects upon the peripheral nerves (ischialic nerves) were foreseen, even in the initial, low numbered animal experiments, which were confirmed by our expanded research. Although, T_{m1} and T_{m2} denaturation temperature maximum values showed a slight change, which proved to be tendentious in the left T_{m1} , however, the thermal domain characterized by T_{m3} showed definitive proof in its significant increase. In the case of peripheral nerves, the overly convincing results are associated with the calorimetric enthalpy calculation in the heat denaturation curve, which in addition to marked dosage dependence, demonstrated a clear and significant decrease compared to the control group. The relatively significant enthalpy change refers to a strong structural alteration, which explains functional damage, considering the marked sensitivity of the nerve tissue.

In the evaluation of the gastrocnemius muscle, significant empirical results were available. The damaging effect of cyclophosphamide is first demonstrated by the changes of T_{m1} and T_{m3} values, in addition to the change of enthalpy, which, once compared with the peripheral nerves, are more moderate, however, represent a one way decreasing tendency. The results support the involvement of the myosin heads and actin filament, on the whole, including the functional damage of the actomyosin complex. It is important to highlight in which the examined actomyosin complex and the enervating ischiadic nerve must be considered to be a functional unit, which is accompanied with a multiplied decrease in function due to the damage.

It should be noted, that the side difference found during the examination of the peripheral nerve and muscle cannot yet be reasonably explained. As long as further investigations and human clinical cases both show a discrepancy of this nature, additional intensive examinations are needed into this direction towards effectively exploring the background.

The denaturation curve of the heart muscle is vastly equaled to the curve of the gastrocnemius muscle, so we experienced similarly valuable information in our examination. In comparing the two muscles, based on the results, we can see, that the heart muscle is sensitive, even to a smaller dose (1-2) treatment, a more significant change is in T_{m1} and T_{m3} denaturation temperature maximum values, while, with an increase in the dosage (3-4) T_{m2} and T_{m3} demonstrate a highly detectable change. The decrease of T_{m2} demonstrates the damage of myosin rods. With the increase of the dosage, calorimetric enthalpy shows an increasing decrease, representing the structural damage. Regarding the entirety of the examination, and, notably, in this particular case, the myosin heads and the actin filament suffered significant damage, however, neither myosin rods were left intact. On the whole,

the functionality of the actomyosin complex, including the pump function, considerably decreases. Structural changes indicated by the calorimetric enthalpy presumably contribute to the development of heart muscle damage, and in this particular case, to the development of hemorrhagic myocarditis.

The effect of a single, small dose of cyclophosphamide injection has the most outstanding beneficial effects on the elements of blood plasma, in which, following the first treatment, a clear increase can be observed at the highest temperatures in heat denaturation. Following a single treatment, and the progression of time, the difference remains, this clearly proves the lasting effect of cyclophosphamide, in which we cannot expect a rapid regeneration from halting the treatment.

In the case of additional treatments, the maximum heat values show a significant decrease, which assumes the damage of biological functions. T_{m1} alterations matching fibrinogen denaturation suggest a fibrinogen dysfunction or a decreased function, thereby demonstrating the damage of the blood clotting system. In clinical practice it forecasts the possibility of hemorrhagic or thromboembolic complications. T_{m2} , opposite with T_{m1} and T_{m3} compared to the control group, significantly increased nearly until the end of the treatment. This difference refers to the functional disorder of albumin, which may influence, among others, the plasma colloid osmotic pressure, the binding and transporting function of the blood, and may decrease the antioxidant effect and the storage of amino acids throughout the body.

When considering the second injection, during repeated treatment, functional damages are strengthened by the increasing calorimetric enthalpy decrease, which demonstrates a higher degree of structural transformation and modification.

In the case of RBC, the results do not show such a degree of a one way tendency, however, fluctuation can be observed in both denaturation maximum temperature values (T_m), and in calorimetric enthalpy differences. The differences are significant in several cases, however, their direction is varies. It can be highlighted, in which the effect of a higher number of treatments (5-6), the number of endothermic reactions continually decreases. Here, functional damage may also occur, in which it likely influences the oxygen transport capacity of the blood, although it is less established, according to the results. To verify this, a well-aimed examination of patients (in human clinical practice) over long term cyclophosphamide treatment is suggested.

Generally, according to the results, the dose dependence of cyclophosphamide induced damage can be stated and supported; however, it is varying in degree and effect depending upon the characteristics of the affected tissue. Strongly expressed alterations are proven on the elements of blood plasma and heart muscle, thus, in all likelihood; the most severe functional alterations can be expected in this respect. There is a definitive effect of damage to both the peripheral nerve and muscle, yet it mostly becomes significant over the long run. Clear, detectable alterations can be observed in the RBC, however, the results are less congruent, and, in this way, their functional manifestation is uncertain yet assuming.

Summary of the thesis

Our study was inspired by the solution of a specific forensic problem in which, under experimental circumstances, we managed to provide a well-founded answer to a question including legal and medical consequences, following chemotherapy.

In taking into consideration the increasing tendency of malignant tumors a more frequent occurrence of side effects due to chemotherapy is certainly expected. Determining the most direct pharmaceutical and medical definition is uneasy and often faces difficulty. Only approximate consequences can be drawn from the administered dosage throughout individuals, due to several reasons in which pharmacokinetics may show individual and unique variations. It is important to emphasize, that therapeutic and toxic levels of most chemotherapeutic agents are associated to one another, and even at time, seemingly overlap. With the help of the DSC examination, the indirect effects can now be detected.

In my own clinical research, the use of interventional onco-radiology during selective transarterial cytostatic treatment and chemoembolization, as well as afterwards, bears a relatively easy determination or estimation of a chemotherapeutic drug level and the systematic effect of the intervention dedicated to be selective, can play an important role. In view of the data, a conclusion can be drawn concerning the predictable effects of the therapy, the success of the intervention and assessment of maintaining the treatment. Predicting the possible side effects and toxic consequences bears the same relevance, which also influences the continual treatment of the patient. Based upon our results, DSC investigation of the blood will provide a distinct possibility for all these.

During the administration of the chemotherapeutic agent chosen for our investigation, cyclophosphamide, clear, detectable dose dependent alterations could be demonstrated both in the case of nerve-muscle complex and in heart muscle and also, in the components of the blood. Synchro-evaluation of our results demonstrates the dosage, when alterations in the components of the blood develop to such an extent which can indicate the damage, with great probability, in the heart muscle or peripheral nerves and gastrocnemius muscle.

Fulfilment of the goals, new result of the thesis

Our investigations well confirmed that during long term administration of cyclophosphamide, we have to count on the development of peripheral neuropathy and motility dysfunction upon the nerve-muscle complex, of which, the background structural modifications can be found.

Definitive structural changes develop within the heart muscle, which, due to deterioration of pump functionality, among others, most likely plays a role in the development of cardiomyopathy, commonly referred to as the complication of cyclophosphamide treatment.

With the help of DSC, the developed, well traceable changes can be seen on the shaped elements of the blood, on RBC and on the blood plasma. The difference in the thermogram illustrates the dose dependence, providing indirect information on the current and cumulative drug levels in the circulation. Additionally, the degree of the confirmed and proven structural changes supposedly correlates with the functional changes and dysfunctions, however, in order to achieve more precise conclusions, further investigations and measurements during human clinical trials are necessary.

DSC detectable alterations have a predictive feature in the assessment of dose dependent side effects and complications.

Based on this, following the sufficient human standardization, DSC may likely be suitable in support of close patient monitoring and to prevent or reduce the potentiality of side effects in daily clinical practice.

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Publications related to the thesis:

Farkas P, Könczöl F, Lőrinczy D: Examination of the peripheral nerve and muscle damage in cyclophosphamide monotherapy with DSC in animal models. *Journal of Thermal Analysis and Calorimetry*. 2016. 126: 47-53.

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Farkas P, Könczöl F, Lőrinczy D: Examination of the left ventricle damage in cyclophosphamide monotherapy with DSC in animal models. *Journal of Thermal Analysis and Calorimetry*. 2017. 127: 1181–1185.

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Farkas P, Könczöl F, Lőrinczy D: Examination of the blood plasma and red blood cells in cyclophosphamide monotherapy with DSC in animal models. *Journal of Thermal Analysis and Calorimetry*. 2017. 127:1239–1243

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Farkas P, Lőrinczy D: New possibilities of application of differential scanning calorimetry - new clinical diagnostic methods on the horizon? *Temperature*. 2017. 4(2): 1-3.

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Lectures related to the thesis:

Farkas P, Könczöl F, **Lőrinczy D:**

Examination of the cyclophosphamide induced polyneuropathy on guinea pig sciatic nerve, gastrocnemius and heart muscle as well as in blood samples with DSC.

Symposium lecture at 12th Conference on Calorimetry and Thermal Analysis and 5th Czech – Hungarian – Polish – Slovakian Thermoanalytical Conference Zakopane, Poland, 2015.

Farkas P, Könczöl F, **Lőrinczy D:**

New Possibilities of application of DSC as a new clinical diagnostic method.

Invited lecture at 1st Journal of Thermal Analysis and Calorimetry Conference and 6th V4 (Joint Czech-Hungarian-Polish-Slovakian) Thermoanalytical Conference Budapest, 2017.