Natural polyphenols improve the dislipidemia and eye complications in the experimental diabetes mellitus

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Abstract
The benefits of the polyphenols extracted from the seeds of black grapes, have been shown by using as experimental model the streptozotocin-induced diabetes mellitus on the Wistar rats. The streptozotocin was administered in a single dose of 60 mg/Kg body mass, intraperitoneal. The vegetal polyphenols were administered under the form of water solution, in a dose of 0,028 g/KG body mass, p.o. (through tube feeding), every two days, for a period of 16 weeks (3 weeks before the streptozotocin induction and 13 weeks after). The results obtained point out a significant improvement (p<0,001) of the lipid profile and a reduced atherogenic risk (the medium value of the atherogenity index obtained at the diabetic rats with polyphenolic protection was of 2.67 in comparison with the diabetic rats, that had an index of atherogenity of 6.23) and an insignificant improvement of the hyperglycemia (p>0.05). All the animals suffering from DM experienced various degrees of retinal detachment. Polyphenols intake by diabetic animals resulted into a low retina disease rate. They experienced only random and uneven retina detachment. Another complication that occurred in all diabetic rats, even in those that received polyphenols treatment, was diabetic cataract. Microscopic investigations reveal a crystalline cortex full of vacuoles or a homogeneous substance with residues of pathological crystalline fibres. Diabetic animals that benefited from polyphenols protection experience only scarce and isolated soft exudates.

Keywords: polyphenols, diabetes mellitus, streptozotocin, oxygen free radicals.

Introduction
The aim of our study was to quantize the effect of natural polyphenols extracted from the seeds of black grapes on the lipid metabolism and the ocular complications in DM.

The DM complication affecting the eyeball may be infectious, chronic or degenerative, and they may diminish eyesight. Chronic degenerative complications, such as diabetic microangiopathy and neuropathy, are closely connected to the disease evolution and duration. Diabetes mellitus (DM) is a metabolic disease in which the dyslipidemia is a major problem [25]. Oxygen free radicals (superoxide anion, hydroxyl radical etc) produced in excess stimulate lipid peroxidation of the polyunsaturated fatty acids forming, thus, in excess, lipid-peroxil radicals. These radicals stimulate in their turn the oxidation of the low-density lipoproteins (LDL) at the level of the vascular intimae, favouring angiopathy [5].

The benefits of moderate red wine consumption are given to a great extent by its specific composition and especially by its content rich in polyphenols [1,2]. The polyphenols are compounds with powerful antioxidant, anti-inflammatory and platelet antiagregant roles [8,30]. The peel and seeds of black grapes contain great quantities of polyphenols that are preserved in the red wine after the manufacturing process [3,4]. The chemically induced
(streptozotocin) DM in rats represents a useful model for the in vivo study of the eye protective qualities of the vegetal polyphenols extracted from the seeds of black grapes. Moderate drinking of red wine has long been known to reduce the risk of cardio-vascular complications [6,10]. This is best known as “the French paradox” [7]. Studies suggest resveratrol in red wine may play an important role in this phenomenon [14, 15]. It achieves the effects by the following functions: (1) Inhibition of vascular cell adhesion molecule expression [11,16]; (2) Inhibition of vascular smooth muscle cell proliferation [22]; (3) Stimulation of endothelial nitric oxide synthase (eNOS) Activity [9]; (4) Inhibition of platelet aggregation [23,29]; (5) Inhibition of LDL peroxidation [12,13,18].

Materials and Methods

a) Polyphenolic extracts from black grapes seeds

The dry extract of vegetal polyphenols was obtained from selected seeds of black grapes from the Station for Wine-Making and Research Copou, Iasi. The extracting method. After the black grapes had been brought to an adequate milling stage, the seeds were extracted with ethyl ether in order to remove the substances with a lipophilic character (volatile oils, greases, waxes etc). The vegetal material was further on treated with ethyl ether [7]. The alcoholic extract was brought to its dry condition at a special temperature (evaporation in normal atmosphere). The obtained solid product had a brown-reddish colour and was brought to a pulverulent state and subjected further on to chemical analyses with a view to determine the total amount of polyphenols. Thus, the total amount of polyphenols determined with the Folin-Ciocalteu reagent may be expressed in rutoside units or gallic acid. The total number of flavonoidic substances expressed in rutoside units was 30.8%, and expressed in gallic acids units 47.6%. Out of the flavonoidic total, 18.3% was represented by the anthocyans. The paper chromatography of the anthocyans (eluent n-butanol: acetic acid: water = 4:1:5, v/v) has shown, through the video-densitometry method, the following composition: 44.9% pelargonidol (Rf=0.29), 36.6%, cianidol (Rf=0.51) and 18.3 % delfinidol (Rf=0.80).

b. The experimental model

The data presented in this article were collected by using the experimental model of streptozotocin-induced diabetes perfected and used within the physiopathology courses at the University of Medicine and Pharmacy “Gr. T. Popa”, Iasi Romania. The research was made on adult white Wistar rats (12 to 14 months), adult, males, with an average weight of 250-280g, which were analyzed in 4 groups of 10 animals.

- **W Group** = witness; rats who were administered 1 ml solution containing 5mL DMSO and 95mL distilled water, every two days (by tube feeding) for a period of 16 weeks
- **DM Group** = rats with diabetes caused by streptozotocin (STZ) injection 60 mg /KG body mass, single dose, intraperitoneal, rapid;
- **P Group** = rats who were administered vegetal polyphenols under the form of solution (in distilled water), in a dose of 0,028g/KG body mass, p.o., (by tube feeding) every two days, for a period of 16 weeks.
- **DM+P Group**= rats who were administered a polyphenolic preparation for 3 weeks before and 13 weeks after the induction of diabetes mellitus.

The diabetes was obtained through the administration of STZ [2-deoxi-2(3-metil-nitrozo-ureido)-p-glucopiranose] in a single dose of 60mg/KG body mass, 1% solution
intraperitoneal (i.p.), after fasting 18 hours. Streptozotocin was used (cytotoxic antibiotic synthesized of *Streptomyces achromogenes*) obtained from SIGMA, S-0130, Lot 31K1379.

The method has the advantage, as compared to the surgical pancreatectomy, of respecting the exocrine pancreas and, consequently of not influencing the digestive processes. The administered dose of vegetal polyphenols (extracted from black grapes seeds) was of 0.028%g/KG body mass every two days, as solution, entherally (by tube feeding). The dry polyphenols extract was diluted in DMSO, 100 ml polyphenolic solution containing 840 mg natural polyphenols, 95mL distilled water and 5mL DMSO.

The animals were kept in normal microclimate conditions. The clinical state of the animals was observed daily, the water and food ingestion, diuresis, glycosuria, and the possible presence of ketone bodies. The animals were fed by a daily ration administration, calculated according to the standard norms of the species. (Nakamura et al. 2002). The rats that showed an altered general state were sacrificed during the experiment, and the other, after 16 weeks from the beginning of the experiment. The blood samples necessary to the biochemical determinations were drawn from the posterior orbital venous sinus.

This study was approved by the Laboratory Animal Care Committee of "Gr. T. Popa" University of Medicine and Pharmacy and the rats were maintained in accordance with the general guidelines for the care and use of laboratory animals recommended by the Council of European Communities. At the end of the experiment, the animals were sacrificed at 12 weeks by cardiac puncture under ketamine anaesthesia (100 mg/kg body weight).

**Parameters of lipid profile**

*Serum total cholesterol, HDL Cholesterol and triglycerides* were measured by enzymatic colorimetric methods on a TECAN micro plate reader by commercially available kits (Audit Diagnostics Ireland). *Non-HDL cholesterol* was calculated by subtracting HDL cholesterol from total cholesterol.

**Microscopic investigations**

- The rat eyeballs were fixed in 10% neutral formaldehyde for 24 hours. Then they were processed using the paraffin baiting technique. We used three stains: haematoxylin and eosin (HE), PAS, Van Gieson and Masson’s Trichrome. The cross sections were examined with an x 10, 20 and 40 lens optical microscope.
- Images of ultra structure were obtained using a Philips CM100 transmission electron microscope (Carl Zeiss Inc., Thronwood, N.Y, U.S.A) and captured with AxioVision software (Carl Zeiss Vision GmbH, Hallbergmoos, Germany).

**Statistical analysis**

Data were expressed as mean ± standard deviation (SD). Statistical significance was determined by variance analysis and one-way ANOVA followed by a post hoc Tukey's test using Statistical Software Package SPSS®, version 13 (SPSS Incorporation, Chicago, IL, USA). Individual comparisons were made using Student’s t test. The effects were considered significant if p<0,005.

**Results**

The dynamic evolution of the glycaemia at the diabetic rats with polyphenolic protection in comparison to the diabetic rats is shown in table I.
Table I: The Glycaemia evolution at the studied groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Average Glycaemia (mg/dL) ± DS</th>
<th>Week 2</th>
<th>Week 10</th>
<th>Week 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>376.55 ±116.82</td>
<td>551.55 ± 166.66</td>
<td>658.44 ± 199.26</td>
<td></td>
</tr>
<tr>
<td>DM+P</td>
<td>329.66 ± 112.07</td>
<td>501.66 ± 172.0</td>
<td>529.44 ± 177.74</td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>73.20 ± 4.30</td>
<td>72.80 ± 4.10</td>
<td>70.80 ± 3.80</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>73.40 ± 4.40</td>
<td>71.80 ± 3.90</td>
<td>70.60 ± 3.70</td>
<td></td>
</tr>
</tbody>
</table>

The hyperglycaemia of the DM+P group was insignificantly reduced (p>0.05) in comparison to the DM group. The normal values are between 50-135 mg/dL, (Pop A, 1996). Thus, the polyphenols administration did not offer protection against the disease installation but the glycaemia evolution was reduced insignificantly (with 19.5% at the diabetic rats with polyphenolic protection in comparison to the rats without protection) [20]. Following the perturbation of the lipid metabolism in the diabetic rats (Table II), Ch-T has significant increased values (p<0.001) in comparison to the rats from W group. The cholesterol increase at diabetic rats may be produced both through the extension of its synthesis (intestinal and hepatic) and the decrease of its degradation. Peripheral and endohepatocytary insulinopaenia is associated with an exacerbation of the HMG-CoA-hepatic reductase activity [26].

Table II: The lipid profile for the adult Wistar rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Ch-T (mg/dL)</th>
<th>TG (mg/dL)</th>
<th>HDL (mg/dL)</th>
<th>LDL (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>74.33±5.61</td>
<td>88.83±7.78</td>
<td>34.17±5.34</td>
<td>24.07±4.69</td>
</tr>
<tr>
<td>P</td>
<td>67 ±2.61</td>
<td>68.33±9.27</td>
<td>31.17±4.88</td>
<td>22.17±4.61</td>
</tr>
<tr>
<td>DM+P</td>
<td>68.83±3.82</td>
<td>91.83±34.8</td>
<td>26.17±3.31</td>
<td>27.83±10.2</td>
</tr>
<tr>
<td>DM</td>
<td>94.5±9.22</td>
<td>152±19.29</td>
<td>17.66±5.66</td>
<td>46.43±7.33</td>
</tr>
</tbody>
</table>

Statistic significance

<table>
<thead>
<tr>
<th></th>
<th>P/W</th>
<th>DM+P/W</th>
<th>DM/W</th>
<th>DM+P/DM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>DM+W</td>
<td>p&gt;0.05</td>
<td>p&lt;0.05</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>DM+P/DM</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>
Thus, one can note that due to the polyphenolic protection of the rats from the DM+P group, the plasmatic Ch-T level is kept at values that may be compared to those of the W group (even slightly diminished) and that are significantly reduced in comparison to the unprotected diabetic group. The positive effect of the polyphenols could be observed also on the not-diabetic animals at which the Ch-T plasmatic concentration was 7.33 mg/dL smaller; insignificant decrease (p<0.05).

At the diabetic rats, HDL has significantly reduced values in comparison to the rats from the W and DM+P group. It is known that in the diabetic states, the reverse transport of cholesterol is often disturbed. The two most frequent met anomalies are: the reduction of HDL-Col and of the HDL₂ fraction [9,11]. The reduction of the HDL fraction may depend directly on the reduction of LCAT (Lecithin Cholesterol Acyltransferase) activity through the functional alteration of ApoA₁ through glycosilation excess [11]. This mechanism is surely responsible by the alteration of the reverse transport of cholesterol in the diabetes mellitus “misbalanced”. In the present experiment it is found that due to the polyphenolic protection of the rats from the DM+P group, the serous level of HDL reaches the normal limits.

**Microscopic investigations**

Diabetic microangiopathy occurs at the retina level, causing diabetic retinopathy (DR), which, given its serious consequences (blindness, significant eyesight reduction). Diabetic retinopathy is the DM complication affecting retina microcirculation, namely capillary, arteriole and venule circulation [19].
Diabetic retinopathy has a natural evolution towards progressive aggravation until complete and irreversible blindness (diabetic cataract), Fig.1a.

All the animals suffering from DM experienced various degrees of retinal detachment (Fig.2a, 3a). This stage is characterized by preretinal and vitreous haemorrhages (4a), retinal tractions, and iris angle neovascularisation. Tissue changes are consequences of alteration of the haemoretinal endothelial barrier (5a) and they consist of soft and hard exudates, haemorrhages, retinal edema, gliosis and mechanical tractions.
Polyphenols intake by diabetic animals resulted into a low retina disease rate. They experienced only random and uneven retina detachment (3b). Dry lipid hard exudates are extravasated lipoprotein deposits through the high-permeability walls of the capillary vessels, located in the outer plexiform retina layer. They usually occur around the macula and consist of hyaline and lipid masses; they may also include erythrocytes. They are a consequence of the chronic retinal edema or of lipid deposits in the blood flow. Diabetic animals that benefited from polyphenols protection experience suffer only small aneurisms (Fig.5b), scarce and isolated soft exudates.

Diabetic animals showed ultra structural damage including intramural pericyte loss in rat retinal capillaries (Fig.6a), disarray of sarcomere, disorganization of mitochondrial [15] matrix (7a), cytoplasm vacuolization, and invagination of nuclear membrane; these were partially normalized by polyphenols treatment (6b, 7b).
Fig. 6a: DM Group - a capillary showing abnormally thickened basement membrane material (EM, x40000)

Fig. 6b: DM+P Group - a capillary with a normal lumen but abnormally thickened basement membrane material (EM, x40000)

Fig. 7a: Group DM - disarray of sarcomere, disorganization of RER (EM, x40000)

Fig. 7b: Group DM+P - cytoplasm vacuolization (EM, x40000)

Discussion

The obtained experimental results are in agreement with the results of other experimental in vivo studies, on diabetic rats that were treated with vegetal polyphenols contained in the red wine [14, 17] or in the green tea [16, 18]. Following the lipid profile it has been found that, streptozotocin-induced diabetes mellitus alters significantly the lipid metabolism at diabetic rats (DM) in comparison to the witness group (W). The administration of vegetal polyphenols extracted from the seeds of black grapes at the diabetic group with polyphenolic protection (DM+P) has significantly ameliorated the lipid profile in comparison to the diabetic group (DM). The mechanisms of resveratrol's (a component of polyphenols) apparent effects on life extension are not fully understood, but they appear to mimic several of the biochemical effects of calorie restriction. Some studies indicate resveratrol activates Sirtuin 1 (SIRT1) and PGC-1α and improves the functioning of the mitochondria [15]. Unfortunately we were not able to purify resveratrol from the polyphenolic extract that we used, in order to study its own effect.

Numerous experimental studies prove the capacity of the vegetal polyphenols to diminish the lipid peroxidation and to reduce the LDL oxidation, probably through the uptake of the lipid-peroxil radicals and through the reduction of the lipoxygenases activity, delaying thus the forming of the atheroma plate [15]. It was found that at the diabetic group with polyphenolic protection (DM+P), the atherogenic risk is strongly diminished due to the increase of the serous concentration of HDL and due to the decrease of the serous level of LDL. The Atherogenity Index is 57.14% smaller than the group DM+P as compared to the MD group [17].

It has been proven that the effect of vascular protection exercised by the polyphenols in the red wine is stronger when the wine is rich in polyphenols. It has been also proven that...
the vegetal polyphenols have the capacity of gathering themselves at the level of the atheroma plate and of stimulating the cholesterol catabolism at the biliary acids, reducing the thickness of the lipid atheroma plate at the hamsters and rats that were subject to a hyperlipemic diet [1,15].

Diabetic microangiopathy occurs at the retina level, causing diabetic DR, which, is characterized by preretinal and vitreous haemorrhages, retinal tractions, traction-related peripheral or retinal detachments and iris angle neovascularisation. The blood presence stimulates the fibroblastic invasion of the vitreous body, and the fibrous tissue thus created subsequently triggers the retina detachment frequently experienced by diabetic animals. The main mechanisms triggering cataract are [28]:

- a higher polyol pathway activity, accompanied by sorbitol accumulations, which barely leave the crystalline lens, thus leading to the latter’s osmotic gradient increase, and determining water accumulation in the crystalline cells, edema and tears causing a higher crystalline lens opacity;
- non-enzymatic glycosylation of the crystalline proteins, which causes the decrease of the crystalline lens transparency;
- a high blood sugar level leading to the formation of excessive free radicals (oxidative stress), and causing irreversible crystalline lens injuries.

Diabetic animals that benefited from polyphenols protection experience only scarce and isolated soft exudates. Polyphenols intake by diabetic animals resulted into a low retina disease rate. They experienced only random and uneven retina detachment. Retina adventitial cells are extremely sensitive to high blood sugar rates, which leads to apoptosis and the loss of their contractile function. Recent studies have proven the existence of anti-adventitial cell antibodies in the serum of DM patients. 

The heart and vessels protective effects of resveratrol are also theorized to be a form of preconditioning—the best method of cardioprotection, rather than direct therapy [21]. A 2011 study [24] concludes, “Our data demonstrate that both melatonin and resveratrol, as found in red wine, protect the heart in an experimental model of myocardial infarction via the Survivor Activating Factor Enhancement (SAFE) pathway, SAFE pathway.”

Studies have shown resveratrol possesses hypoglycaemic and hypolipidemic effects in both streptozotocin (STZ)-induced diabetes rats and STZ-nicotinamide-induced diabetes rats. Resveratrol ameliorates common diabetes symptoms, such as polyphagia, polydipsia, and body weight loss [20]. Other diabetic animal model studies by different researchers have also demonstrated the antidiabetic effects of resveratrol [27]. The effects of the Grape polyphenols extract favour also better insulin sensibility.

We can draw the conclusion that the polyphenolic extract, with a flavonoidic content of 30.8% in rutoside units out of which 18.3% are anthocyanins (44.9% resveratrol, 36.6% cianidol, 18.3% delfinidol), obtained from seeds of black grapes, administered at diabetic rats in a dose of 0.028 k/Kg, for 16 weeks, improves significantly (p<0.001) the lipidic profile, reduces significantly (p<0.001) the atherogen risk and improves diabetic retinopathy. In future, we plan to investigate the effect of the same polyphenols extract on microangiopathy in DM nephropathy and maybe to prove the same beneficial role on a different pathway of DM progression.

Conclusions

This study emphasizes the improvements in the lipid and carbohydrate levels in diabetic rats that enjoyed natural polyphenols protection. The histological pictures of diabetic rat retina, sclera and crystalline lens reveal significant alterations. The most frequent one is retina...
detachment. Diabetic rats with polyphenols intake experienced significantly less severe retinal conditions, except for crystalline lens opacity, which was constantly frequent in all of them.

To conclude with, a proper blood sugar level control and a lower dyslipemia may reduce and even prevent eye complications in general and diabetic retinopathy in particular. The protection provided by vegetable polyphenols in experimental DM depends both on the amount of the polyphenols intake and on the duration of their intake.

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