

Protective effects of polyphenolics in red wine on diabetes associated oxidative/nitrative stress in streptozotocin-diabetic rats

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Abstract

Increased accumulation of NT (3-nitrotyrosine) and PARylated [poly(ADP-ribose)ylated] proteins in the tissues of diabetics are associated with diabetes complications (diabetes neuropathy, nephropathy and retinopathy). Red wine (its polyphenols are considered to be the main active components) can act as ROS (reactive oxygen species) scavengers, iron chelators and enzyme modulators. This study is novel in investigating the effect of red wine in preventing the accumulation of NT and PARylated proteins in the sciatic nerve, DRG (dorsal root ganglia), spinal cord, kidney and retina of diabetic animals. We have shown that during the experiment the body weight of control and diabetic groups of rats with consumption of red wine was significantly increased, by 52% and 19% accordingly. The significant increase in the content of NT in the sciatic nerve, DRG, spinal cord, kidney and retina, and PARylated proteins in the sciatic nerve, renal glomeruli and retinae of diabetic rats was partly or completely prevented by treatment with red wine. Red wine and its polyphenol preparations might be a promising option in the prevention and treatment of diabetic complications.

Keywords: diabetes mellitus; poly(ADP-ribose) polymerase; peroxynitrite

1. Introduction

Diabetes mellitus has reached epidemic proportions – 3–6% of the population of developed countries are suffering from the disease. Hyperglycaemia is the main diagnostic index, irrespective of diabetic type and mechanisms, i.e. AGE (advanced glycation end-product) formation (Nogueira-Machado and Chaves, 2008; Negre-Salvayre et al., 2009), aldose reductase activity (Drel et al., 2006a), accumulation of ROS (reactive oxygen species) and highly reactive oxidant peroxynitrite (a product of superoxide anion radical reaction with nitric oxide) (Chander et al., 2004; Pacher et al., 2005), activation of PKC (protein kinase C) and hexosamine pathway (Schleicher and Weigert, 2000; Gálvez, 2009), and triosophosphate accumulation (Wahlberg et al., 2000) can lead to diabetic complications.

The important role of NT (3-nitrotyrosine), a footprint of peroxynitrite-induced injury and other reactive nitrogen species, activation of PARP [poly(ADP-ribose) polymerase] in chronic diabetic complications is well established (Brownlee, 2005; Obrosova et al., 2005; Pacher et al., 2007). Accumulation of NT and poly(ADP-ribose) – the product of PARP-catalysed reaction in response to DNA damage has been reported in peripheral nerve (Obrosova et al., 2005; Drel et al., 2006b), DRG (dorsal root ganglia) neurons (Obrosova et al., 2007), kidney (Xiao et al., 2009) and retina (Drel et al., 2009) of Type 1 and Type 2 diabetic rats and mice. Alleviation of nitrative stress and inhibition of PARP resulted in the reversal of functional changes in diabetic neuropathy, nephropathy and retinopathy in STZ (streptozotocin)-diabetic rats and mice, as

well as NOD (non-obese diabetic) mice (Nangle et al., 2004; Sugawara et al., 2004; Obrosova et al., 2007; Chandak et al., 2009; Drel et al., 2009).

Polyphenolic compounds, i.e. anthocyanins, resveratrol, gallic acid, catechin, myricetin, quercetin, etc. that are abundant in the red wines, could play a major role in enhancing the antioxidant system, since they behave as ROS scavengers, metal chelators and enzyme modulators (Lodovici et al., 2001). Polyphenols could enhance antioxidant capacity of plasma in humans due to their chelating properties by avoiding free radical formation through Haber–Weiss/Fenton reactions (Duthie et al., 1998; Tsang et al., 2005), participate in the regulation of vascular tone and inhibit platelet aggregation (Dell'Agli et al., 2004).

However, the role of red wine in the prevention of diabetes-associated nitrative stress has been poorly analysed. The present study was designed to evaluate the effect of red wine on nitrative stress and PARP activation in sciatic nerve, DRG neurons, spinal cord, kidney and retinae of STZ-diabetic rat. The possible physiological significance of these findings is discussed.

2. Materials and methods

2.1. Chemicals

The STZ and reagents were of reagent-grade quality and were purchased from Sigma Chemical Co. Rabbit polyclonal anti-nitrotyrosine antibody was purchased from Upstate and mouse

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Abbreviations: AGEs, advanced glycation end products; DAB, 3,3'-diaminobenzidine; DRG, dorsal root ganglia; NT, 3-nitrotyrosine; PARP, poly(ADP-ribose) polymerase; PKC, protein kinase C; ROS, reactive oxygen species; STZ, streptozotocin.

monoclonal anti-poly(ADP-ribose) antibody was purchased from Trevigen, Inc. Secondary Alexa Fluor 488 goat anti-rabbit and Alexa Fluor goat anti-mouse antibodies as well as Prolong Gold Antifade Reagent were purchased from Invitrogen. Avidin/Biotin Blocking Kit, VECTASTAIN Elite ABC Kit, DAB (3,3'-diaminobenzidine) Substrate Kit, were obtained from Vector Laboratories. The red wine used in the study was kindly donated by the Institute of Vine and Wine "Magarach".

2.2. Animals

All animal care and procedures were in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes Directive of 24 November 1986 (86/609/ECC) and were approved by Bioethics Committee of Ivan Franko National University of Lviv Protocol for Animal Studies, Lviv, Ukraine. Male Wistar rats, of 120–150 g body weight, were fed a standard rat chow and had access to water *ad libitum*.

2.3. Experimental protocols

Wistar rats were separated into four groups of seven animals in each: Group 1 – normal untreated control; Group 2 – red wine treated; Group 3 – STZ treated and Group 4 – red wine and STZ treated. The STZ treatment was a single i.p. injection of 50 mg/kg body weight. The red wine treatment was an oral dose (300 ml/70 kg body weight/day) administered daily for 2 weeks prior to the STZ injection and daily for 4 weeks after the STZ injection. Group 2 received red wine for 6 weeks.

The red wine was made by the classical technology from Cabernet Sauvignon grapes and contained phenolic compounds 2309.31 mg/l, proanthocyanidines 936.0 mg/l and pigment polymers 443.8 mg/l.

Blood samples for glucose measurements were taken from the tail vein ~72 h after the STZ injection and the day prior to the study termination. All rats with blood glucose of 14 mmol/l or more were considered diabetic.

2.4. Anaesthesia, euthanasia and tissue sampling

The animals were sedated by CO₂ and immediately killed by cervical dislocation. One sciatic nerve, kidney, eye and parts of the spinal cord and DRG (lumbar and sacral nerve roots) from each rat were fixed in 10% neutral-buffered formalin for further assessment of poly(ADP-ribose) by conventional immunohistochemistry. The second sciatic nerve, kidney, eye and another part of the spinal cord and DRG from each rat were immediately frozen in liquid nitrogen for Western blot analyses of protein NT content.

2.5. Western blot analyses of nitrosylated proteins

To assess nitrosylated proteins by Western blot analysis, tissue samples were transferred to an extraction buffer (1:10 wt/vol) containing 50 mM Tris/HCl, pH 7.2; 150 mM NaCl; 0.1% SDS; 1% NP-40; 5 mM EDTA; 1 mM EGTA; 1% sodium deoxycholate and the protease/phosphatase inhibitors: leupeptin (10 µg/ml),

aprotinin (20 µg/ml), benzamidine (10 mM), phenylmethylsulfonyl fluoride (1 mM), sodium orthovanadate (1 mM) and homogenized on ice. The homogenate was sonicated (3 × 5 s) and centrifuged at 14000 g for 20 min. All the aforementioned steps were done at 4°C. The lysates (20 µg of total protein per lane) were mixed with an equal volume of 2 × sample-loading buffer containing 62.5 mM Tris/HCl, pH 6.8; 2% SDS; 5% 2-mercaptoethanol; 10% glycerol and 0.025% Bromophenol Blue, heated at 95°C for 5 min, separated on 10% SDS/PAGE and transferred onto a nitrocellulose membrane (Towbin et al., 1992).

Free binding sites were blocked in 2% (w/v) BSA in PBS containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.7 mM KH₂PO₄, pH 7.3, and 0.05% Tween 20 for 1 h, after which nitrotyrosine antibodies were applied for 2 h. The horseradish peroxidase-conjugated secondary antibody was applied for 1 h. After extensive washing, protein bands detected by the antibodies were visualized with the ECL (enhanced chemiluminescence) Detection Reagents (Amersham). The total content of all nitrosylated proteins was quantified by densitometry (Gel Pro Analyzer 3.1, Media Cybernetics). Membranes were stripped in 25 mM glycine-HCl, pH 2.5 buffer containing 1% SDS and reprobed with β-actin antibody to confirm equal protein loading.

2.6. Immunohistochemical studies

All sections were processed by a single investigator and evaluated blindly. Poly(ADP-ribose) immunoreactivities in the sciatic nerve, spinal cord, and DRG neurons, kidney and retina were assessed by immunohistochemical techniques (light and fluorescent microscopy). Tissues fixed in 10% neutral-buffered formalin were processed through to paraffin embedding, and 7 µm sections were cut. The sections were deparaffinized in xylene, hydrated in decreasing concentrations of ethanol and washed in water. Optimal staining was achieved with antigen retrieval solution.

For immunofluorescent histochemistry, non-specific binding was blocked with 10% of normal goat serum and 1% BSA in PBS buffer in a humidity chamber for 1 h. Mouse monoclonal anti-poly(ADP-ribose) antibody was diluted 1:100 in 1% BSA in PBS and applied overnight at 4°C in the humidity chamber. Secondary Alexa Fluor 488 goat anti-mouse antibody was diluted 1:200 in PBS and applied for 2 h at room temperature. Sections were mounted in Prolong Gold Antifade Reagent.

For light microscopy, endogenous peroxidase was quenched with 0.3% H₂O₂ for 20 min. Non-specific binding was blocked with the 10% of normal goat serum and 1% BSA in PBS buffer in the humidity chamber for 1 h. Avidin/biotin blocking kit (Vector Laboratory Inc.) was used to block endogenous biotin and avidin, and mouse monoclonal anti-poly(ADP-ribose) antibody (diluted 1:100 in 1% BSA in PBS) was applied overnight at 4°C in the humidity chamber. Detection was performed using secondary biotin-conjugated goat anti-mouse antibody (diluted 1:200 in PBS) applied for 2 h at room temperature and a Vectastain Elite ABC kit (Vector Laboratory Inc.). Positive signals were visualized with the DAB Substrate Kit (Vector Laboratory Inc.). Sections were counterstained with haematoxylin, dehydrated and mounted in the Micromount mounting medium (Surgipath Medical Ind., Inc.).

Negative controls included elimination of the primary antibody. At least 10 fields of each section were examined to select one representative image. Representative images were microphotographed, and the intensity signal was quantified with ImageJ 1.32 software (National Institutes of Health). Low-power observations of sciatic nerve, spinal cord, DRG, kidney and retina sections stained for poly(ADP-ribose) were made using a Nikon Optiphot 2 imaging microscope. Colour images were captured with a DCM310 microscope CMOS camera. Low-power images were generated with a $\times 40$ acroplan objective using the automatic capturing feature of the ScopePhoto software.

2.7. Statistical analysis

Data are expressed as mean \pm S.E.M. Differences among experimental groups were determined by ANOVA (analysis of variance), and the significance of between-group differences assessed by Student–Newman–Keul’s multiple range test. Significance was defined at $P \leq 0.05$.

3. Results

Initial (after STZ injection) blood glucose concentration was 133% higher in diabetic rats compared with controls (Table 1). Hyperglycaemia progressed with the prolongation of diabetes, and the difference between final blood glucose concentrations in the two groups exceeded 3-fold. Red wine consumption did not affect blood glucose concentrations in control or diabetic rats.

Initial body weights were similar in control and diabetic rats. The final body weights were lower by 36% in the diabetic group compared with control (133.4 ± 8.2 compared with 208.8 ± 18.9 g, $P < 0.01$) (Table 1). This increase was slightly, but significantly, increased by the consumption of red wine (171.8 ± 10.9 g, $P < 0.05$ compared with control, and $P < 0.05$ compared with untreated diabetic group).

Sciatic nerve, DRG, spinal cord, renal parenchyma and retina nitrosylated protein abundance assessed by Western blot analysis was increased by 48%, 60%, 40%, 52% and 38% correspondingly in the untreated diabetic group compared with the control group (Figures 1A–1J), and such increases were essentially or completely prevented in diabetic rats treated with red wine ($P < 0.05$, $P < 0.01$, $P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively, compared with untreated diabetic group).

Table 1 Initial and final body weights and blood glucose concentrations in control and diabetic rats with or without red wine (RW) consumptions. Results are expressed as means \pm S.E.M., $n = 5-7$.

Rodent group	Body weight (g)		Blood glucose (mmol/l)	
	Initial	Final	Initial	Final
Control	136.6 ± 15.5	208.8 ± 18.9	6.1 ± 0.8	6.1 ± 1.3
Control+RW	138.8 ± 12.4	203.4 ± 18.4	6.3 ± 0.9	6.5 ± 0.9
Diabetic	141.0 ± 11.7	$133.4 \pm 8.2^{**}$	6.0 ± 1.3	$23.2 \pm 2.4^{**}$
Diabetic+RW	144.0 ± 13.9	$171.8 \pm 10.9^{*\#}$	6.1 ± 1.0	$24.4 \pm 4.8^{**}$

* $P < 0.05$ and ** $P < 0.01$ compared with controls.

$P < 0.05$ compared with diabetic rats without wine consumption.

Poly(ADP-ribose) immunofluorescence was increased by 21% in the sciatic nerve of diabetic rats compared with the control group (Figures 2A and 2B), and this increase was significantly reduced ($P < 0.01$ compared with untreated diabetic group) by red wine consumption. DRG and spinal cord poly(ADP-ribose) fluorescence was not different among the experimental groups (microphotographs represent only untreated control and diabetic groups) (Figures 2C, 2D and 2E).

Poly(ADP-ribose) immunoreactivities were increased in glomeruli of the renal cortex by 28.5% and in retina by 34.5% of diabetic rats compared with non-diabetic controls, an increase that was markedly reduced by red wine treatment (Figures 2F–2I). Poly(ADP-ribose)-positive nuclei were localized primarily in the ganglion cell layer, but were also detectable in other parts of the retina.

4. Discussion

One of the well-recognized fundamental mechanisms in the pathogenesis of chronic diabetes complications is an oxidative–nitritative (also known as nitrosative) stress, resulting from increased production of ROS (Pacher et al., 2007). In diabetic and hyperglycaemic conditions, superoxide, as a primary free radical, is rapidly converted to several other ROS, i.e. hydrogen peroxide, hydroxyl radicals and peroxynitrite. Peroxynitrite causes numerous cytotoxic effects (nitritative stress), i.e. protein nitration with resultant changes in cell signalling (activation of mitogen-activated protein kinases (Pesse et al., 2005), the nuclear transcription factor NF- κ B (Yakovlev et al., 2007), up-regulation of TGF- β (transforming growth factor- β) (Sugiura et al., 2006), vascular endothelial growth factor (el-Remessy et al., 2005); DNA single-strand breakage and base modification; overactivation of PARP with resultant NAD⁺ depletion and energy failure (Pacher et al., 2007), changes in transcriptional regulation and gene expression, with concomitant activation of several major pathogenetic mechanisms implicated in diabetic chronic complications (Brownlee, 2005).

Our results provide evidence of clearly manifest nitritative stress in sciatic nerve, DRG neurons, spinal cord, kidney and retina under diabetic conditions. We found the increased accumulation of NT (a marker of peroxynitrite-induced injury) in the tissues of STZ-diabetic rats is consistent with elevated level of nitrated protein content (assessed by immunohistochemistry) in other reports (Kowluru et al., 2007). Furthermore, accumulation of NT has recently been documented in vascular endothelium, myocardium, retina, kidneys and peripheral nervous system of diabetic rodents (Obrosova et al., 2005; Obrosova et al., 2007; Drel et al., 2009), as well as in obese fatty Zucker rats, Zucker diabetic fatty rats and leptin knockout (ob/ob) mice (Chander et al., 2004; Drel et al., 2006b). It was also shown that multiple tissues of human subjects with diabetes, i.e. circulation, vasculature, kidneys, skin and myocardium accumulate NT (Ceriello, 2002; Hoeldtke et al., 2003; Santilli et al., 2004). These data indicate that peroxynitrite-induced injury is presented in early and late stages of Type 1 and Type 2 diabetes mellitus, and during the prediabetic stage.

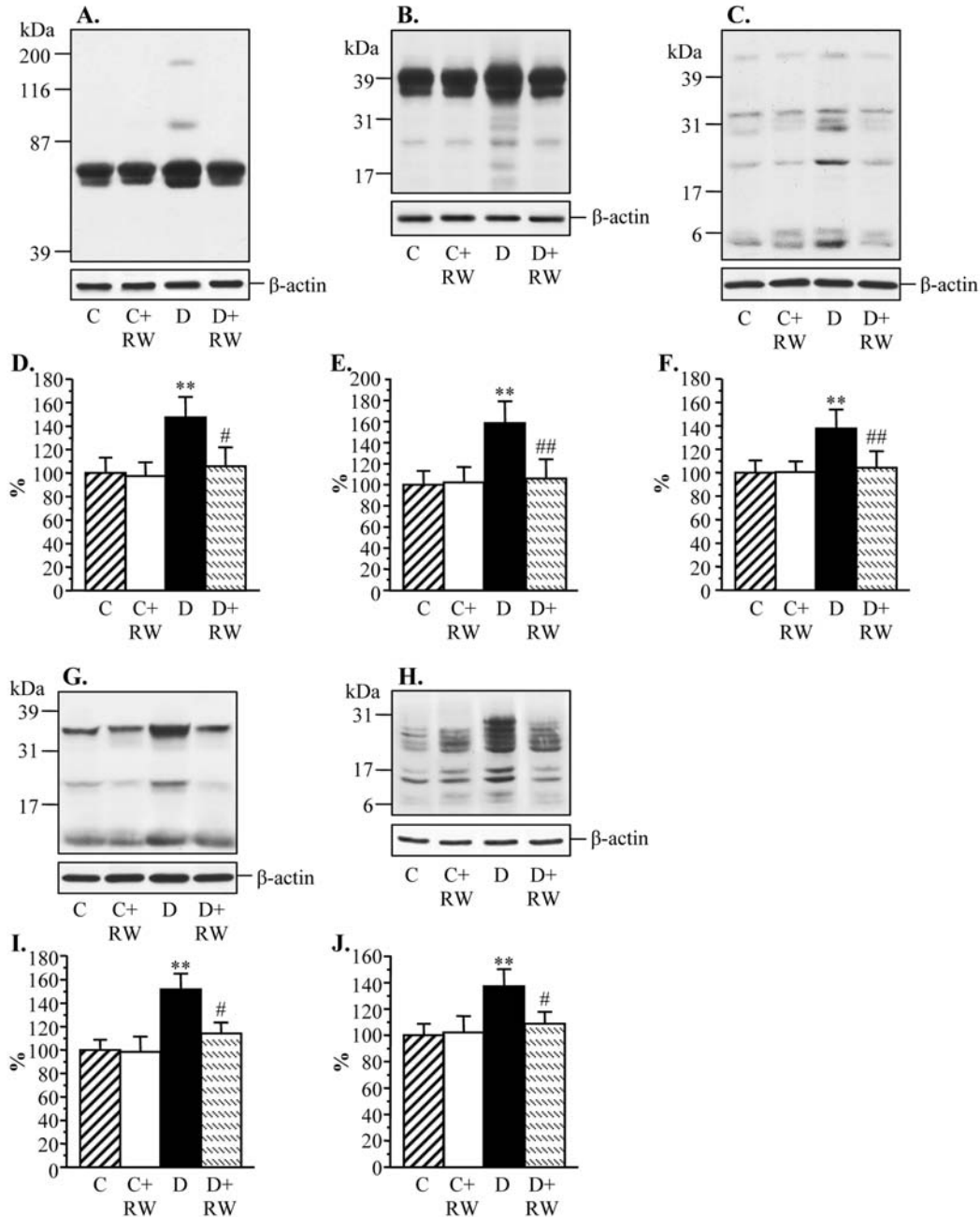


Figure 1 Assessment of nitrotyrosine accumulation in the tissues of control and diabetic rats with and without red wine consumption, using Western blot analysis

Representative Western blot analyses of nitrotyrosine-modified proteins in the sciatic nerve (A), DRG (B), spinal cord (C), renal parenchyma (G) and retinae (H) of control and diabetic rats with and without wine consumption. Total nitrotyrosine content (D), (E), (F), (I) and (J), respectively. Total nitrotyrosine protein content in control rats is taken as 100%. Equal protein loading was confirmed with β -actin antibody. C, control groups; D, diabetic groups; RW, red wine. The data are expressed as means \pm S.E.M., $n=5-7$. ** $P<0.01$ compared with controls. # $P<0.05$ and ## $P<0.01$ compared with diabetic rats without wine consumption.

Our results suggest that red wine treatment has a substantial effect on lowering the NT content in sciatic nerve, DRG neurons, spinal cord, kidney and retina of diabetic rats. This may be due to red wine-mediated decrease in oxidative stress. Free radicals react with phenolic compounds much faster than with proteins, lipids or DNA; therefore, phenols can protect proteins, lipids and DNA from oxidative damage.

We have also shown clear manifestation of poly(ADP-ribose) accumulation in sciatic nerve, renal glomeruli and retina. Moreover, red wine reduced the level of poly(ADP-ribosyl)ated proteins in diabetic animals to the control level. Oxidative-nitrosative stress and PARP overactivation are interrelated with several other hyperglycaemia-initiated mechanisms. The accumulation of sorbitol and fructose induced by hyperglycaemia

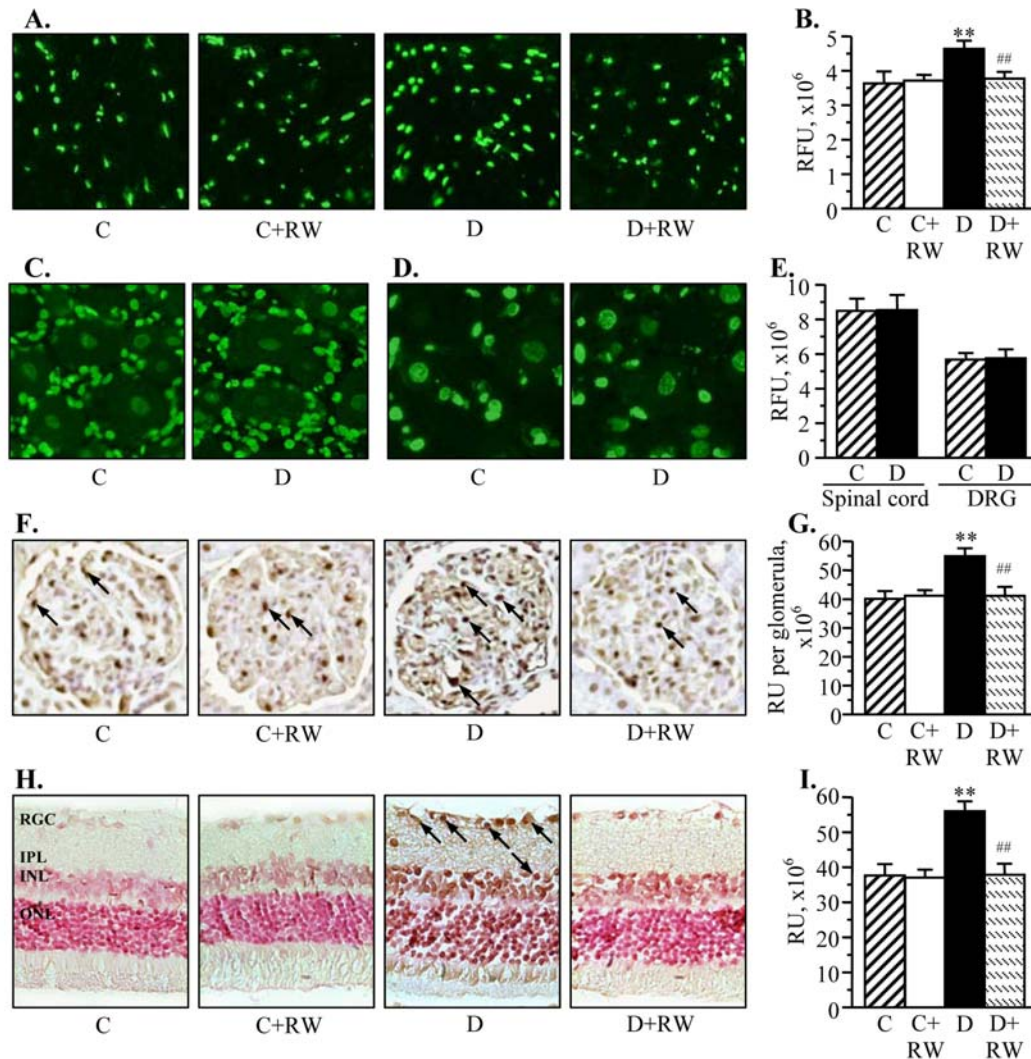


Figure 2 Immunohistochemical assessment of PARYlated [poly(ADP-ribose)ated] proteins in the tissues of control and diabetic rats with and without red wine consumption

Representative microphotographs of immunofluorescent staining of poly(ADP-ribose) in sciatic nerves (A), DRG (C) and spinal cord (grey matter within the dorsal horn) (D) of control and diabetic rats with and without wine consumption. Intensity of poly(ADP-ribose) fluorescence [relative fluorescence units (RFU) per image] in sciatic nerves (B), DRG and spinal cord (E). Magnification $\times 100$. Microphotographs of light microscopy immunohistochemical staining of poly(ADP-ribose) in renal glomeruli (F) and retinae (H) of control and diabetic rats with and without wine consumption. Magnifications $\times 80$ and $\times 40$, respectively. Total poly(ADP-ribose) content (relative units per image) in renal glomeruli (G) and retinae (I). Arrowed examples of poly(ADP-ribose)ated proteins of renal glomeruli and retinae cells, stained in dark brown colour. RGC, retinal ganglion cells; IPL, inner plexiform layer; ONL, outer nuclear layer; INL, inner nuclear layer. Means \pm S.E.M., $n=10-15$ per group. C, control rats; D, diabetic rats; RW, red wine. $n=10-15$. ** $P<0.01$ compared with controls; ## $P<0.01$ compared with diabetic rats without wine consumption.

leads to the formation of AGEs, which in turn generate free radicals during interaction with their receptors (Yamagishi, 2009). Peroxynitrite-induced DNA breakage overactivates PARP, leading to poly-(ADP-ribose)ation of nuclear proteins and resulting inhibition of the glycolytic enzyme, glyceraldehyde 3-phosphate dehydrogenase, accumulation of methylglyoxal, sorbitol and fructose, with concomitant activation of several major pathogenetic mechanisms, i.e. non-enzymatic glycation, activation of PKC and the hexosamine pathway (Brownlee, 2005; Kanwar and Kowluru, 2009). Activation of PKC and increased AGEs formation can trigger various inflammatory processes that can damage blood vessels.

The absence of overactivation of PARP in DRGs and spinal cord, compatible with our previous study (Drel et al., 2009) and due to axonal degradation under pathological conditions, begins from the most distal part of the nerve (Dobretsov et al., 2007). Therefore, PARP overactivation could act not only as an early marker of oxidative-nitrative damage, but also as a marker of the next stage of diabetes-mediated pathological changes in peripheral neuropathy.

Apart from showing protection from oxidative-nitrative stress and activation of PARP, our data also show that treatment with red wine increases the body weight of diabetic rats. Excess glucose production and low glucose utilization in the body raises

levels of blood glucose (hyperglycaemia), which leads to increased osmotic diuresis that is quickly followed by fluid loss and electrolytes and ultimately dehydration. An increase in free fatty acids during diabetes, in turn, leads to the production of ketoacids in the liver with the concomitant over-acidic condition of metabolic acidosis (Dunger et al., 2004). All the above-mentioned conditions lead to a decrease in body weight and other diabetes complications. The effect of red wine (weight loss alleviation) may be due to its positive effect on kidney homeostatic functions and/or protection from ketoacids, but further studies are required to validate this assumption. Moreover, other studies showed that quercetin and resveratrol (flavonoids widely present in red wines) administration to rats reversed back to normality the glucose tolerance test, hepatic glucokinase and hexokinase activities and the lipid profile; it can also improve the antioxidant status in different tissues, such as liver, kidney, brain and heart (Palsamy and Subramanian, 2008; Bournival et al., 2009). All these data support our findings, suggesting the protective action of red wine and its 'polyphenolic acid'.

5. Conclusions

Our results provide the rationale for further studies of detailed mechanisms of anti-diabetic effects of red wine. Understanding the mechanism involved in the action of red wine can have potentially profound clinical implications.

Author contribution

Nataliya Sybirna conceived and designed the study. Viktor Drel carried out the experimental procedures. Nataliya Sybirna and Viktor Drel drafted the manuscript, and read and approved the final manuscript.

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