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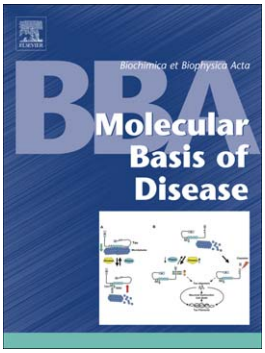

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Effects of rosiglitazone on intramyocellular lipid accumulation in *Psammomys obesus*

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Abstract

Objective: To examine the effects of rosiglitazone in intramyocellular lipid (IMCL) content in diabetic *P. obesus* using novel electron microscopy technologies.

Background: *P. obesus* is an unique polygenic model of obesity and type 2 diabetes. Male diabetic *P. obesus* were treated daily with 5 mg/Kg Rosiglitazone by oral gavage for 14 days. Data were compared with a group of age-matched diabetic *P. obesus* treated with saline vehicle.

Methods: Assesment of insulin resistance and adiposity were determine before and after the treatment period by oral glucose tolerance test (oGTT) and dual energy X-ray absorptiometry (DEXA) analysis. *We* used a new scanning electron microscopy technology, (WETSEM) to investigate the effects of rosiglitazone administration on IMCL content, size and distribution in red gastrocnemious muscle.

Results: Rosiglitazone treatment improved glucose tolerance in *P. obesus* with no difference in the overall body fat content although a significant reduction in subscapular fat mass was observed. Rosiglitazone changed the distribution of lipid droplet size in skeletal muscle. Treated animals tended to have smaller lipid droplets compared with saline-treated controls.

Conclusions: Since smaller IMCL droplets are associated with improvements in insulin sensitivity, we propose that this may be an important mechanism by which rosiglitazone affects glucose tolerance.

Keywords: intramyocellular lipid, rosiglitazone, thiazolidinedione, *Psammomys obesus*

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Introduction

Thiazolidinediones (TZD's) such as rosiglitazone improve whole body insulin sensitivity in both rodents [1, 2] and humans with insulin resistance [3, 4]. TZD's act in a range of tissues to improve insulin sensitivity including adipose tissue [5], skeletal muscle [1, 6, 7], liver [1, 6] and macrophages [8]. TZD's are also thought to have protective effects in pancreatic β-cells [9]. The principle mechanism of action of TZD's is thought to be through agonism of peroxisome proliferator-activated receptor-gamma (PPARγ), leading to transcriptional regulation of a number of genes in various tissues. Collectively, these transcriptional changes are thought to cause a re-distribution of lipids from skeletal muscle and liver to adipose tissue. Together with increased adipogenesis, this results in increased storage of lipids in smaller, more insulin sensitive adipocytes, leading to an overall improvement in insulin sensitivity [10, 11].

Recent studies have demonstrated the importance of inflammation in the development of insulin resistance [12-14]. TZD's have been shown to have significant anti-inflammatory effects in a range of in vitro and in vivo models, and in human subjects [15-18], leading to some conjecture regarding the principal mechanism of action by which TZD's improve insulin sensitivity.

Accumulation of triglycerides in skeletal muscle is strongly associated with insulin resistance, and IMCL content is associated with both insulin resistance and type 2 diabetes [6, 19-23]. However a paradox has been described whereby highly trained endurance athletes have high levels of IMCL but are very healthy and highly insulin sensitive [24-26]. It appears that it is not only the amount of lipid in skeletal muscle but the size and distribution of the lipid droplets that determines the level of insulin sensitivity in this organ.

While it is clear that the accumulation of IMCL is strongly associated with insulin resistance and the risk of type 2 diabetes, the effects of rosiglitazone on IMCL levels have been inconsistent. Several studies have shown that rosiglitazone reduced IMCL in conjunction with improved insulin sensitivity [27-31], however a number of other studies have found no change [32] or an increase in IMCL following treatment with rosiglitazone [33, 34].

The aim of the current study was to examine the effects of rosiglitazone treatment in *Psammomys obesus*, a polygenic animal model of obesity and type 2 diabetes. In their natural semi-arid habitat, these gerbil-like rodents feed on low energy plants such as saltbush (*Atriplex halimus*). However, when fed a high caloric diet in captivity, they develop aspects of the metabolic syndrome characterised by obesity, insulin resistance and hyperglycaemia.[35, 36]. To address the aim of this study we have utilised a novel electron microscopy technology to investigate the effects of rosiglitazone on IMCL content, size and distribution in skeletal muscle.

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Materials and Methods

Animals

Male *P. obesus* were fed a standard rodent diet (12% of energy from fat, 63% from carbohydrate and 25% from protein, 3.58 Kcal/g; Barastoc, Pakenham, Australia) after weaning (4 weeks of age) that induced hyperglycaemia (blood glucose > 8 mmol/L) in ~15% of the animals at 16 weeks of age. Animals were housed in a temperature-controlled room (22±1°C) with a 12-12 h light-dark cycle. Animals were maintained in accordance with the Code of Practice of the National Health and Medical Research Council of Australia, and all procedures were carried out subject to the approval of the Deakin University Animal Welfare Committee.

Study protocol

Two experimental groups (saline or rosiglitazone treated) of 16-20 week-old animals were matched for body weight, food consumption and circulating glucose and insulin levels (see table 1). Both groups of animals were hyperinsulinemic but normoglycaemic at the beginning of the study. Each group were treated by daily oral gavage of rosiglitazone (5 mg/kg) or saline (control) for 14 d. Body weight and food intake were measured weekly. Blood samples were collected for assessment of circulating glucose and insulin levels on days 0, 7 and 14. At the start and end of the study, all animals had energy homoeostasis assessed by indirect calorimetry (24 h), and body composition measured by DEXA analysis. An oral glucose tolerance test (2 g/kg glucose) was performed on day 14.

WETSEM technology: background

Red gastrocnemius muscle was harvested and immersed in formalin, and longitudinally cut to a thickness of 0.5-1 mm, and stained with phosphtungstic acid (PTA; Sigma, St. Louis, MO), which highlights proteins and membranous structures. Using this staining methodology, intracellular features were highlighted brightly whereas lipid droplets remained unstained in black.

The WETSEM technology is especially useful to resolve lipid compartments in the cell, based on their rich carbon content compared with other cellular structures richer in oxygen atoms. The WETSEM technique was described recently in detail [37-39]. Briefly, each stained muscle section was absorbed onto a thin partition membrane transparent to electrons inside a sample capsule (QuantomiX Ltd. Nes Ziona, Israel) and placed on the specimen stage of the SEM under vacuum. Images were obtained on an FEI XL-30 (Eindhoven, The Netherlands) scanning electron microscope (electron beam energy of 15-30 KV, beam current of 200-800 pA, with scan rates of 1.3-120 msec/line at 484 lines/frame).

Assessment of lipid accumulation in muscle

For each animal, a total of 40 fibers were imaged, representing different longitudinal muscle fibers per sample. Determination of IMCL droplet size and total amount was performed using custom-made in-house software (QuantomiX, Nes Ziona, Israel). Typical resolution of choice for image processing was as shown in Figure 3.

Statistical analysis

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The relationship between the dependent variable from the IMCL data, and several independent variables from the blood biochemical data was analyzed by multiple regression analysis using SAS software.

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Results

Effects of rosiglitazone in *Psammomys obesus*

Treatment with rosiglitazone had no effect on body weight, percentage body fat (assessed by DEXA) or plasma insulin concentration in *P. obesus* (Table 1). However, rosiglitazone treatment caused a significant reduction in mean blood glucose concentration (p=0.006), and was associated with improved glucose tolerance as measured by OGTT (Figure 1). During the OGTT, blood glucose concentrations were significantly lower in rosiglitazone treated *P. obesus* compared with controls after 45 min (p= 0.04), 60 min (p=0.04) and 90 min (p=0.01), consistent with an improvement in glucose tolerance following treatment with rosiglitazone. Furthermore, after 14 days of rosiglitazone treatment (5 mg/kg/d), the area under the glucose curve in an OGTT was reduced by 19% (p=0.039) compared with controls (Figure 1B).

There was no difference in the weights of major organs (liver, heart, pancreas, spleen, heart, adrenal gland, and testis) in the rosiglitazone treated animals compared with saline treated controls. Consistent with the results of the DEXA analysis of body fat content, the combined weight of the various adipose tissue depots was not different between the groups (rosiglitazone 12.0±1.2 vs. saline 14.7±1.7 g, p=0.20). In the animals treated with rosiglitazone, the weight of the sub-scapular (subcutaneous) fat pad was reduced by 38% when compared with the saline treated control animals (Figure 2, p=0.025). This difference remained when the sub-scapular fat pad mass was expressed as a percentage of body weight (rosiglitazone 1.24±0.11 v saline 1.92±0.27 % of body weight, p=0.020).

There was no significant difference in the mass (or percentage of body weight) for any of the other adipose depots tested (peri-renal, epididymal, mesenteric or intramuscular; Figure 2), nor for any of the skeletal muscles measured (data not shown).

Treatment of *P. obesus* with rosiglitazone had no effect on total energy expenditure, physical activity, or whole-body fat or carbohydrate oxidation as measured by indirect calorimetry in *P. obesus*.

Imaging and quantitative analysis of IMCL

Figure 3 shows a series of representative muscle images taken at different magnifications, using the WETSEM technique. The high-resolution images of the muscle fibers, showing the precise structure of the striated skeletal muscle, the IMCL droplets and the mitochondria, enabled determination of IMCL droplet size.

Total IMCL content per fibre area was not significantly different between the treatment groups, although IMCL tended to be lower in the rosiglitazone treated animals (Fig. 4A). Analysis of IMCL droplet size distribution indicated a pattern of reduced lipid droplet size in the skeletal muscle of *P. obesus* treated with rosiglitazone

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(shown in black bars) compared with control animals (white bars; Fig. 4B). There was significantly reduced incidence of larger lipid droplets in the rosiglitazone treated animals, relative to the controls (χ^2=9.9, $p<0.05$).

Correlation between physiological parameters and IMCL analysis

We used multiple regression analysis to investigate relationships between physiological data and the IMCL data set obtained from the WETSEM technique. When all animals were analysed together, a combination of the area under the curve of the OGTT results (OGTT-AUC) and the percentage body fat at day 14 was significantly associated with IMCL droplet size (R^2 = 0.44). Within the saline treated animals, OGTT-AUC (p=0.02) and fat content at day 14 (p=0.009) were strongly and independently associated with IMCL droplet size (R^2 = 0.74).

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Discussion

In this study we have shown that treatment with rosiglitazone (5 mg/kg) for 14 days significantly improved glucose tolerance in *P. obesus*. The improvement in glucose tolerance was not associated with a change in total IMCL, but there was a trend for a reduction in lipid droplet size in skeletal muscle following rosiglitazone treatment. Furthermore, we demonstrated that IMCL was significantly associated with glucose tolerance and overall body fat content. Collectively, these data suggest that the effect of rosiglitazone on glucose tolerance may be mediated in part by changing the distribution of lipid droplet size in skeletal muscle, even if there is no overall reduction in total IMCL.

Treatment with rosiglitazone did not affect body weight or body fat content in *P. obesus*. However, the mass of the sub-scapular fat pad was reduced in the treated animals compared with controls. There was no difference between the groups for any of the other fat pads measured, and the relevance of this finding to the overall effects of rosiglitazone on glucose tolerance is unknown. However, this finding does provide additional evidence to the suggestion that treatment with rosiglitazone results in re-distribution of lipids between tissues.

The relationship between IMCL and insulin resistance is well established [19-23]. However, the effects of rosiglitazone on IMCL are less clear [32-34], and there is some conjecture regarding the mechanisms by which this drug improves insulin sensitivity. Here we provide evidence to suggest that rosiglitazone treatment results in a reduction in lipid droplet size in skeletal muscle without a significant change in total IMCL. Hefetz and colleagues also reported no change in the total lipid content in skeletal muscle from diabetic *P. obesus* following rosiglitazone treatment, although they did not examine the size distribution of the lipid droplets [40]. Interestingly, microscopy analysis of pancreatic sections revealed that rosiglitazone prevented the decrease in insulin immunostaining induced by the high energy diet and accelerated β-cell proliferation (measured by BrdU incorporation into nuclei) [40]. Based on these observations, Hefetz et al. concluded that the major anti-diabetic action of rosiglitazone in *P. obesus* was by preventing the deterioration of pancreatic function. While we have not formally examined insulin content in β-cell islets in this study, we observed no change in circulating insulin levels after rosiglitazone treatment. This suggests that the beneficial effects of rosiglitazone on glycaemia and glucose tolerance shown in this study are not likely due to an increase in insulin secretion. The alternative conclusions reached in both studies may be due to differences in the dose of rosiglitazone and the high calorie feeding protocol used.

We believe that a reduction in lipid droplet size in skeletal muscle may be an important factor in the improvement in glucose tolerance observed in these animals Consistent with this hypothesis, Fraenkel et al showed that a decrease in the number of large lipid droplets in skeletal muscle was associated with reduced hyperglycaemia when high energy diet induced-diabetic *P. obesus* were switched to a low energy diet [41]. Likewise, He and colleagues [42] showed a profound increase in insulin sensitivity following exercise training and weight loss in obese subjects, with no change in total IMCL. However the size of intramuscular lipid droplets was significantly reduced and this reduction was correlated with insulin sensitivity [42]. One potential mechanism by which a reduction in lipid droplet size may be associated

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with insulin sensitivity independent of total IMCL is through an increased surface to volume ratio of the droplets, which increases the proportion of PAT proteins (perilipin, adipophilin, and TIP47) relative to triglyceride content, in turn improving the access of cytosolic lipases to the lipids [43]. It has also been suggested that smaller lipid droplets are more accessible to oxidation by mitochondria [42].

The improvement in glucose tolerance with reductions in lipid droplet size but no change in total IMCL is also consistent with the "athletes paradox". Highly trained endurance athletes have high levels of IMCL but are highly insulin sensitive [24-26]. These observations have been explained by the fact that oxidative capacity is greatly increased following exercise training, although the precise role of lipid droplet size and subcellular location have not been fully explored. Our results, along with those of others [42] suggest that lipid droplet size may be an important determinant of insulin sensitivity of skeletal muscle, and that this is one mechanism by which rosiglitazone can affect glucose tolerance.

In summary, rosiglitazone improved glucose tolerance in *P. obesus*, and this effect was associated with a re-distribution of lipid droplet size in skeletal muscle. We hypothesise that this may an important aspect of the mechanism of action of rosiglitazone, and could explain some of the inconsistent results seen previously regarding the effects of rosiglitazone on IMCL. Future studies should include assessment of not only total IMCL, but also the size of the lipid droplets in skeletal muscle.

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Figure Legends

Figure 1: A) OGTT blood glucose data showing that rosiglitazone improves glucose tolerance in *P. obesus*. *$p<0.05$ compared with saline treated control animals. B) Area under the blood glucose curve (OGTT) data showing an improvement in glucose tolerance following rosiglitazone treatment in *P. obesus*. *$p=0.039$.

Figure 2: Fat pad weights from *P. obesus* treated with rosiglitazone or saline (control). *$p=0.025$.

Figure 3: Wet SEM images of skeletal muscle from *P. obesus* treated with rosiglitazone (C and D) or saline (controls; A and B). Scale bars are shown in the lower right corner of each image.

Figure 4: A) Total IMCL per fibre area in *P. obesus* treated with rosiglitazone and saline. B) IMCL droplet size distribution (normalised to the mean per 10 fibres) in rosiglitazone (black bars) and saline (control; white bars) treated *P. obesus*. $\chi_2=9.9$, $p<0.05$.

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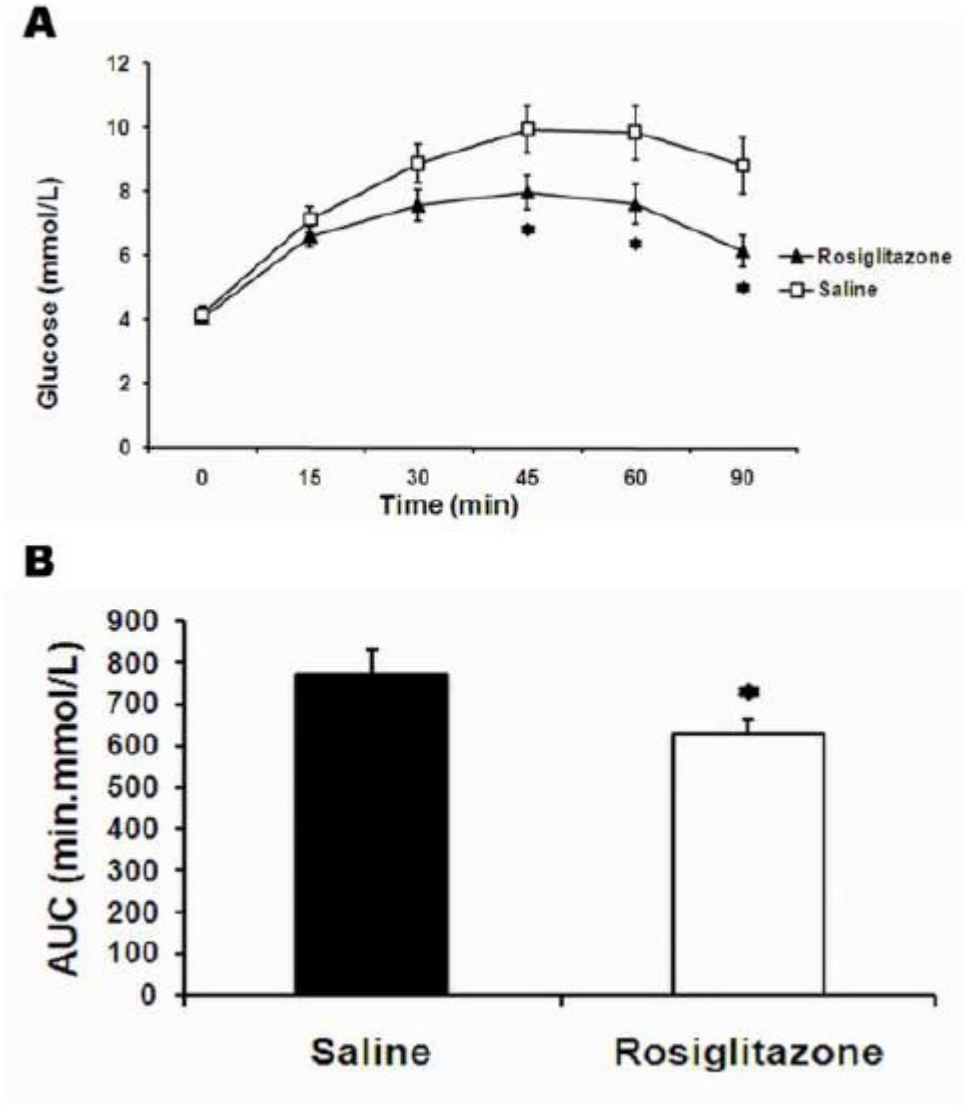

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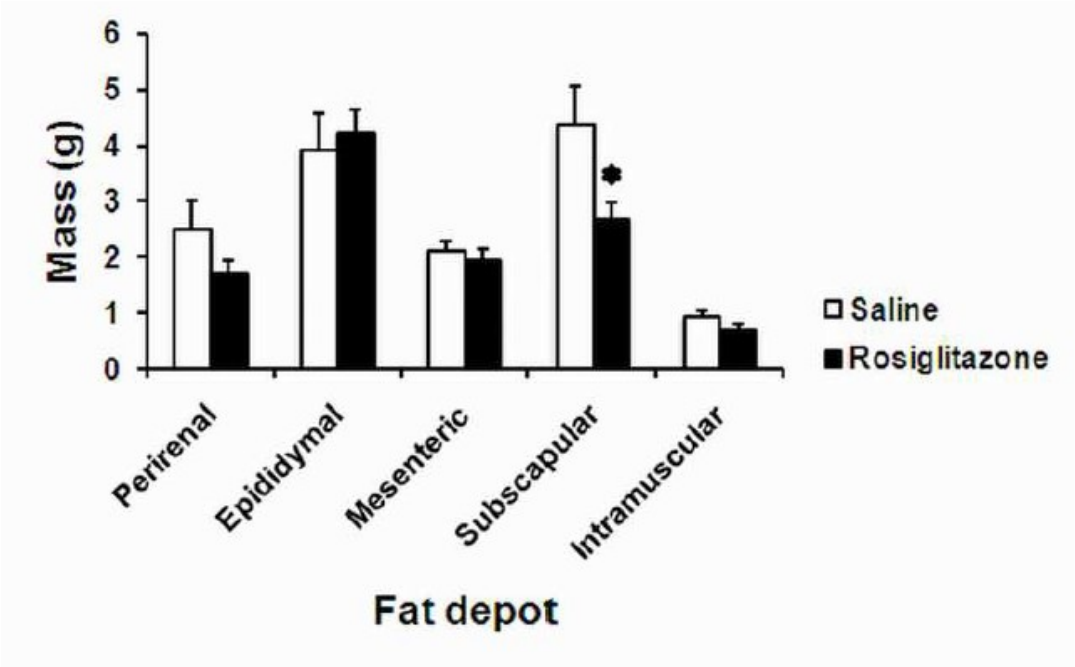

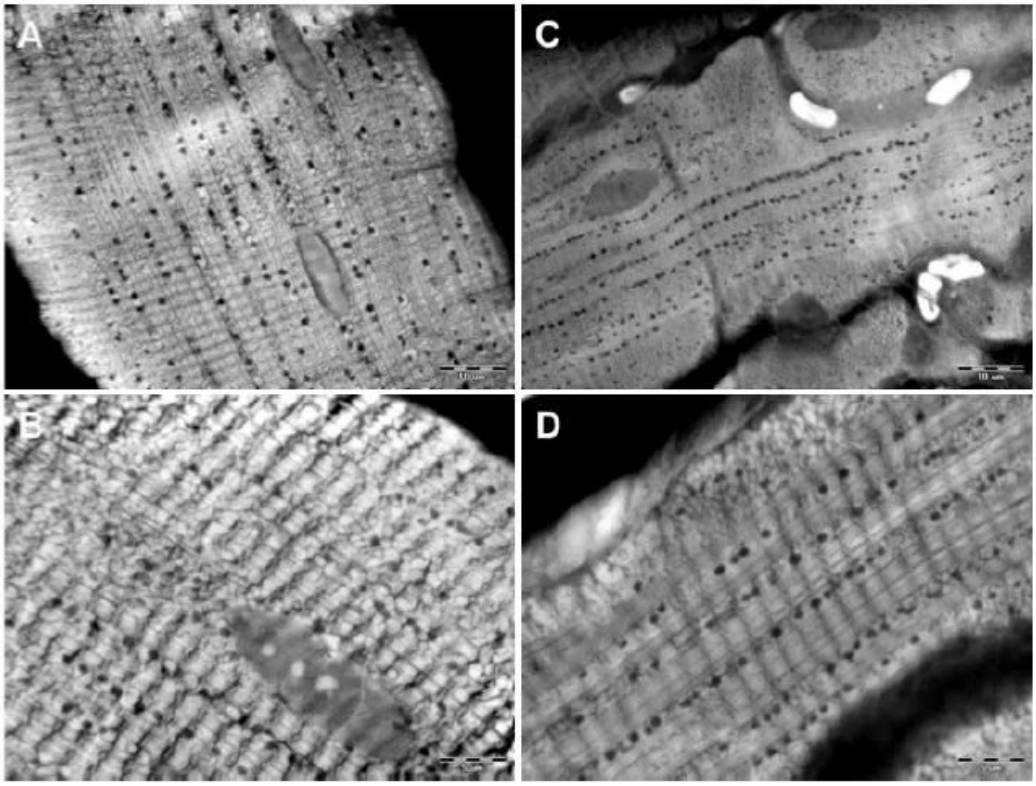
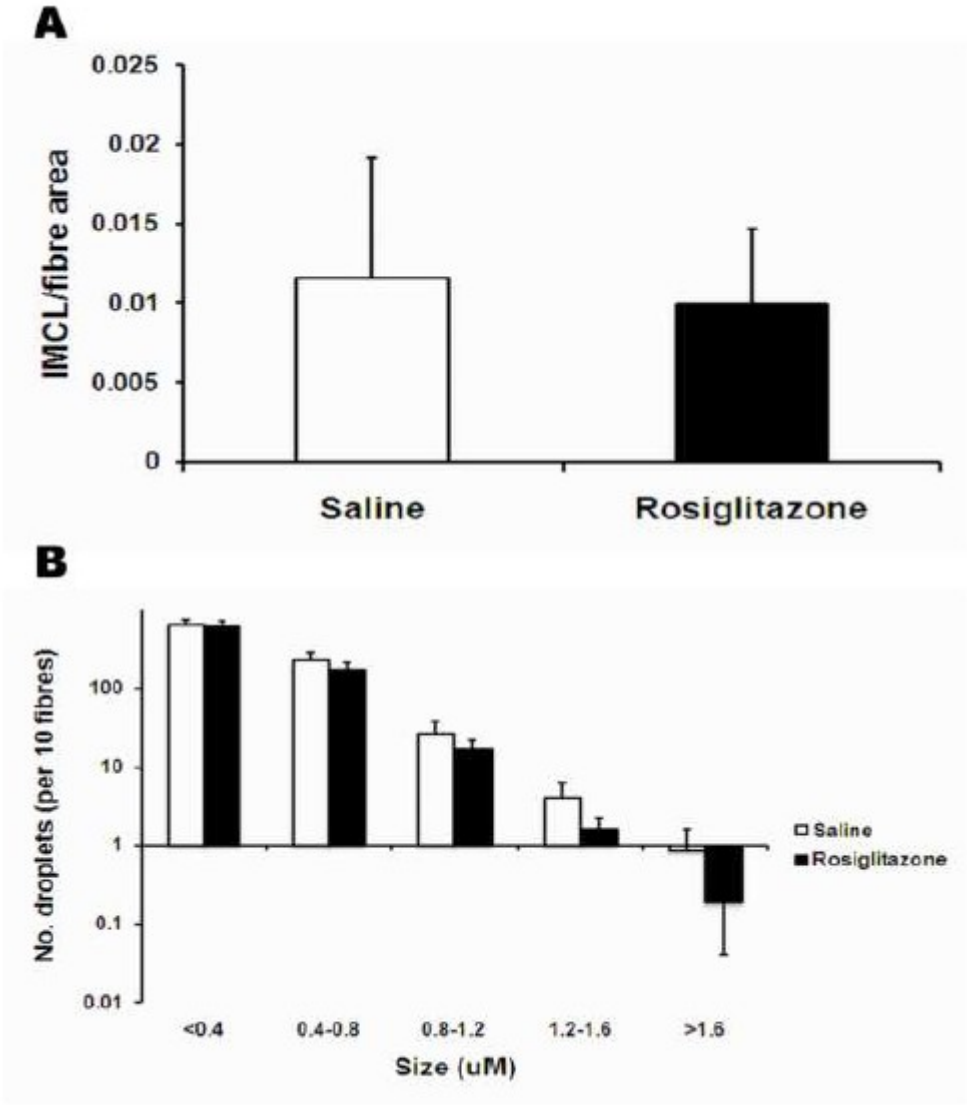
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