Title: Caveolin: a possible biomarker of degradable metallic materials toxicity on vascular cells.

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Keywords: Degradable metals; fibroblasts; smooth muscle cells; caveolin; cytotoxicity

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Abstract: Iron-based materials could constitute an interesting option for cardiovascular biodegradable stent application due to their appropriate ductility compared to their counterparts namely, magnesium alloys. However, the predicted degradation rate of pure iron is considered too slow for such application. We explored manganese (35% w/w) as an alloying element in combination with iron to circumvent this problem through powder metallurgical process (Fe-35Mn). Manganese, on the other hand, is highly cytotoxic. We recently explored a new method to better characterize the safety of degradable metallic materials (DMMs) by establishing the gene expression profile (GEP) of cells (mouse 3T3 fibroblasts) exposed to Fe-35Mn degradation products in order to better understand their global response to a potentially cytotoxic DMM. We identified a number of up- and down-regulated genes and confirmed the regulation of a subset of them by quantative RT-PCR. Caveolin-1 (cav1), the structural protein of caveolae little plasma membrane smooth invaginations present in various differentiated cell types, was one of the most down-regulated genes in our GEPs. In the present study, we further studied the potential of this 22kDa protein to become a biomarker for cytotoxicity towards the exposure of degradable metallic elements. In order to better characterize cav1 expression in this context, 3T3 mouse fibroblasts were exposed to either ferrous and manganese ions at a cytostatic concentrations for 24 or 48 hours. Cav1 gene expression was not influenced by exposition to ferrous ions. On the other hand, manganese exposure for 24 hours reduced cav1 gene expression by about 30% and >65% at 48 hours compared to control 3T3 cells. Cav1 cellular protein content was also reduced to the same extent. The same pattern of expression for cav3 (the muscle-specific caveolin subtype) was also observed in the study. This strong and reproducible pattern of regulation of caveolins thus exhibits a potential as a biomarker for the toxicity of DMM elements.
Reviewer #3: This revised manuscript has been improved substantially based on the reviews' suggestion, however, there are a few concerns that should be addressed before acceptance:

Major concern:

1. Regarding the newly added western blot images, the authors should consider including blot images of endogenous gene/protein (GAPDH) along with the images of interested proteins (cav1, cav3) since the former was used as loading control to ensure that the same amount of total protein was added in each well in electrophoresis. 
   
   GAPDH images were added to the figures.

2. In addition, without provision of blot images of your endogenous control and normalization of interested proteins to endogenous control, it's hard to correlate the protein band with the protein histograms (such as in Fig2 (24h), Fig 5 (48h), Fig 6 (48h), Fig 7 (24h)).
   
   The blot below the histogram is one of 6 from which the histograms are made. This is hard sometimes to find one that looks exactly like the histogram for each figure. In addition, normal variations between bands in the GAPDH control will only add to the problem in our opinion. The image of a blot is indicative of our data but the histogram is the true representation of the data.

Minor concern:

1. In methods section, "western blotting" part, since the authors provided detail information (product information and dilution times) about antibodies against cav1 and cav3, similar information should be provided regarding the remaining antibodies (anti-alpha-SMA and anti-GAPDH).
   
   Since the clone number of the antibodies were provided and their dilution in the manuscript, we are not sure what additional information the reviewer wants. We did add the dilution for the other two antibodies used. We do not feel that our results would be influenced by an different incubation time, a dilution of primary antibody, the use of a different secondary antibody or different amounts of crude homogenate migrated on a gel since the control for each experiment appears on the blot and we do not compare blots probed with different antibodies, experiments made for different times or in different cell types between each other.

2. In order to avoid confusion, the authors should use the same pattern to fill the histogram bar for Fe and Mn in Fig 1 and Fig 2.
   
   We made the changes.

3. In Fig 3 legend, the authors should indicate how many times the experiments were repeated.
   
   We added a mention about this in the legend.

4. In Fig 6 legend, the 3rd sentence, "Fibroblasts" should be changed to "smooth muscle cells".
5. In Fig 7 and Fig 8 legends, the first sentence, "protein content in A10 smooth muscle cell line", one "in" should be deleted.

OK

6. As there are two No. 12 references, I'm wondering if this mistake will cause misconceptions regarding context and references.

References were re-numbered.
Caveolin: a possible biomarker of degradable metallic materials toxicity in vascular cells.

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Iron-based materials could constitute an interesting option for cardiovascular biodegradable stent application due to their appropriate ductility compared to their counterparts namely, magnesium alloys. However, the predicted degradation rate of pure iron is considered too slow for such application. We explored manganese (35% w/w) as an alloying element in combination with iron to circumvent this problem through powder metallurgical process (Fe-35Mn). Manganese, on the other hand, is highly cytotoxic. We recently explored a new method to better characterize the safety of degradable metallic materials (DMMs) by establishing the gene expression profile (GEP) of cells (mouse 3T3 fibroblasts) exposed to Fe-35Mn degradation products in order to better understand their global response to a potentially cytotoxic DMM. We identified a number of up- and down-regulated genes and confirmed the regulation of a subset of them by quantative RT-PCR. Caveolin-1 (cav1), the structural protein of caveolae little plasma membrane smooth invaginations present in various differentiated cell types, was one of the most down-regulated genes in our GEPs. In the present study, we further studied the potential of this 22kDa protein to become a biomarker for cytotoxicity towards the exposure of degradable metallic elements. In order to better characterize cav1 expression in this context, 3T3 mouse fibroblasts were exposed to either ferrous and manganese ions at a cytostatic concentrations for 24 or 48 hours. Cav1 gene expression was not influenced by exposition to ferrous ions. On the other hand, manganese exposure for 24 hours reduced cav1 gene expression by about 30% and >65% at 48 hours compared to control 3T3 cells. Cav1 cellular protein content was also reduced to the same extent. The same pattern of expression for cav3 (the muscle-
specific caveolin subtype) was also observed in the study. This strong and reproducible pattern of regulation of caveolins thus exhibits a potential as a biomarker for the toxicity of DMM elements.

Keywords: Degradable metals, fibroblasts, caveolin
Introduction

Caveolae, little caves, were first described as flask-shaped plasma membrane invaginations capable of transporting molecules across the endothelial barrier. They are found abundantly mostly in highly differentiated cell types such as endothelial and smooth muscle cells [1]. Caveolae have been associated with a number of cellular functions or processes such as potocytosis, cholesterol homeostasis, transformation, and the control of signal transduction [2-3]. Their functions are related to their structure, rich in cholesterol, sphingomyelin and glycosphingolipids. Caveolins, the biochemical marker for caveolae structures, also play a central structural role in their formation. The caveolin family comprises at least three genes that are expressed heterogeneously in different cell types. Caveolin-3 (cav3) is specifically expressed in muscle cells [4-5], while caveolin-1 (cav1) and caveolin-2 (cav2) are expressed in most cell types such as fibroblasts, adipocytes, endothelial cells, and pneumocytes [6].

The implantation of a metallic stent in an artery is associated with a mechanical disruption of the endothelial layer. Using biodegradable metallic stents could also exert additional damages to the surrounding tissue via the release of degradation end-products. Caveolin 1 has recently been reported as a potential biomarker of vascular injury caused by certain vasodilators [7] Cav1 is highly expressed in normal blood vessels and its expression is decreased at the site of damage in vascular injury. This raises the possibility that cav1 expression could act eventually as a marker of vascular injury at the site of implantation of a stent. Cav1 also acts as a binding site of nitric oxide synthase (NOS) which is responsible for nitric oxide production [8].
We recently reported that caveolin-1 expression was decreased in a cultured fibroblastic cell line when exposed to the degradation products of degradable metallic materials (DMMs) [9]. In fact, earlier experiments with 3T3 fibroblast cells showed that cav1 was down-regulated in the presence of DMM and that this pattern of expression was observed as early as 24-hour exposures with Fe-35Mn alloy and was stable up to 48-hour exposures. It is generally believed that the presence of DMMs is associated to the release of metallic ions that act as oxidants and lead to an imbalanced charge within the surrounding tissue [10]. Our findings showed that the expression of cav1 was decreased in the presence of DMMs which is in accordance to other reported results where ozone was applied as an oxidant in lung-injury mouse model [11]. In this study, we studied further the expression pattern of caveolins when cells are exposed to DMMs.
Materials and methods

Cell culture

BALB/3T3 mouse fibroblast cells (ATCC number CL-163, Clone A31) were cultured in Dulbecco’s Modified Eagle’s Medium (Invitrogen, Burlington, Canada) supplemented with 10% fetal bovine serum (Thermo Scientific, UT) and 1% antibiotic/antimycotic (Thermo Scientific, UT) at 37°C in a humidified incubator at 5% CO2. A10 (ATCC number CL-163, Clone A31) rat smooth muscle cells, SMCs, were cultured in M199 culture medium (Invitrogen, Burlington, Canada) in the same incubation condition to that of fibroblasts. For experiments involving cell exposure to the DMMs degradation products, cells were plated in 24-well cell cultures plates at a density of 100cells/µl and 60cells/µl for fibroblasts and SMCs respectively. Cells were then left overnight to adhere to the culture plates.

Metal powders of pure iron, pure manganese, and the alloy were used. Powders of pure iron and manganese were obtained from GoodFellow Inc. (Oakdale, PA), while powder of the alloy was prepared by mechanical filing and sieving that produced alloy particles less than 75 µm. Powders were chosen in order to create an optimum condition of high surface area in contact with culture medium. Cytostatic amounts of metal powders in 500µl of culture medium were poured in tissue culture inserts (3.0µm, Corning, NY) and placed above the fibroblasts monolayer. After 24 or 48 hours of incubation, the tissue culture inserts were removed and the remaining medium was aspirated. The wells were then rinsed with PBS 1X and the cells were trypsinized and counted using haemocytometer.
In experiments using salts of iron (FeCl2) and manganese (MnCl2), the salts were directly solubilised into cell culture medium without the utilisation of culture inserts. At the end of incubation period, the cells were counted in the same manner as described above.

In certain experiments, the cellular metabolic activity was measured as previously described [10]. After the 24-hour incubation the culture medium was removed and 250 µl DMEM with 10 % (v/v) WST-1 (Sigma, Oakville, ON, Canada) was added to each well for two hours at 37°C. Finally, 100 µl of medium from each well was transferred into a 96-well plate and a colorimetric measurement was performed on a spectrophotometer at 450 nm.

RNA extraction and Quantitative RT-PCR

Total RNA was extracted from 6 different replicates for each condition using Trizol following supplier’s protocol (Invitrogen, Burlington, ON, Canada). Total RNA samples were diluted to 500 ng/µl as described elsewhere [12]. One-microliter of RNA was subsequently converted to cDNA using the QuantiTect Reverse Transcription kit (Qiagen, Valencia, ON, Canada). The resulting cDNA was then diluted 10-fold with water prior to amplification (final concentration corresponding to 5 ng/µl of initial RNA). Five microliters of diluted cDNA were amplified (n=2) by qRT-PCR in a Rotor-Gene thermal cycler (Corbett Life Science, Sydney, Australia) using QuantiTect Primer Assays and QuantiFast SYBR Green PCR kits (Qiagen, Valencia, ON, Canada). To correctly judge the efficiency of the amplification reactions, a no-template control was applied to
each run which included both a tube of water only as well as a series of three 10-fold dilutions of the representative cDNA. Quantification of gene expression level was based on the 2-ΔΔCt method [13]. The mean of threshold cycle (Ct) values of duplicates for each particular gene were then subtracted by the mean Ct value (hence ΔCt) of the control housekeeping gene cyclophilin a (PPIA). The difference in the mean Ct values between groups of treatments (Ct) allows for the calculation of the relative levels of expression of particular genes.

Western blotting

Cultured cells were collected using trypsin/EDTA and were pelleted by centrifugation. The pellet was then homogenized in the following lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EGTA, 20 mM β-glycerophosphate, 1% NP-40, 10 mM NaF, 2 mM Na3VO4 and a cocktail of protease inhibitors) and was subsequently transferred to sodium dodecyl sulfate (SDS)-polyacylamide gel electrophoresis. Moreover, the gel was blotted to nitrocellulose membranes, blocked for 1 h and then incubated for overnight at 4 °C with primary antibodies mouse monoclonal cav1 (clone 2297) or cav3 (clone 64) antibodies (1:1000; BD Biosciences, Mississauga, ON, Canada). Alpha smooth muscle actin antibody and for GAPDH ware from Sigma (1:1000). Chemiluminescent bands were visualized and quantified with a ChemilImager system (Alpha Innotech Corporation). Densitometry values for the 22-24kDa band corresponding to caveolins 1 or 3 were expressed as a ratio for GAPDH (antibody from
Sigma) protein content and expressed in arbitrary units on the graphs. Controls were set to 1.

Statistical analysis

Other results are presented as mean ± SEM unless specified otherwise. Inter-group comparisons were done using one-way ANOVA and Tukey’s post-test. Statistical significance was set at a p<0.05. Data and statistical analysis were performed using Graph Pad Prism version 5.01 for Windows, Graph Pad Software (San Diego, CA).
Results

DMMs down-regulate caveolin expression in 3T3 fibroblasts.

We chose to conduct the experiments described here at a “cytostatic” dose of a given material or salt. We defined a cytostatic dose as sufficient to slow proliferation of the cells and to avoid massive cellular death impairing the capacity to perform expression studies. In practice, we performed our incubation at a dose of materials where after 24 hours; the total viable cell number was equal to the one put in culture at the beginning. Previous studies showed that the cytostatic dose of the Fe35Mn alloy (alloy) and manganese in the form of powder is 5 mg/ml of culture medium in the insert and 0.25 mg/ml respectively. As for the iron powder, up to 32 mg/ml did not give any significant effect to 3T3 fibroblast cells [14]. As illustrated in Figure 1, caveolin-1 (cav1) mRNA levels were strongly down regulated in presence of manganese powder or the alloy after 24 hours. Iron powder (32 mg/ml) did not changes cav1 gene expression. A similar pattern was observed after 48 hours of incubation. We then checked if the total Cav1 protein content followed the same type of regulation as for the mRNA in 3T3 fibroblasts. As illustrated in Figure 2, the results obtained by immunoblotting using a specific Cav1 antibody on total cellular homogenates separated on SDS-PAGE, were comparable to those of Cav1 gene expression.

Since we were not able to achieve a cytostatic concentration using iron powder, we used FeCl$_2$ and MnCl$_2$ salts. The salts are soluble within the cell culture medium providing free metallic ions. These experiments were thus conducted without culture inserts. As shown in Figure 3, following a 24-hour incubation period, we determined that a concentration of 0.15 mg/ml of FeCl$_2$ or 0.025 mg/ml of MnCl$_2$ achieved the desired
cytostatic effect in 3T3 fibroblasts. This time, when treated with this cytostatic concentration of FeCl$_2$, caveolin-1 mRNA levels (Fig. 4) and protein content (Fig. 5) were both decreased similarly to those treated with the manganese salt.

*DMMs down-regulate caveolin expression in A10 smooth muscle cell line.*

The main artery wall constituent responsible for restenosis after stenting is the smooth muscle cells. Although smooth muscle cells express a certain amount of caveolin-1 (data not shown), the main caveolin is isoform 3. We used A10 cells, derived from the thoracic aorta of embryonic rat and a commonly used model of vascular smooth muscle cells. As for the fibroblasts, exposition to A10 cells to metal powder (4 mg/ml of the alloy and iron powders and 0.25mg/ml of manganese powder) reduced drastically caveolin 3 protein content (Fig.6) as well as caveolin 3 gene expression (not shown). We determined that concentrations as small of 0.1 mg/ml of FeCl$_2$ and 0.05 mg/ml of MnCl$_2$ gave a cytostatic effect to A10 cells (not shown). At these concentrations, total protein content of caveolin-3 was decreased by more than 50% compared to controls (Fig. 7). We then test if of metal salts exposure modified the expression of the alpha smooth muscle actin ($\alpha$-SMA), a marker for smooth muscle cell After 48 hour exposure to metal salts, the expression of the $\alpha$-SMA remained unchanged (Figure 8).
Discussion

Our results showed that both cav1 and cav3 was down regulated in the presence of metal elements both in the forms of powder or salt.

DMMs have been proposed for some specific biomaterial applications, including paediatric, orthopaedic and cardiovascular applications. DMMs are designed to disappear via corrosion once the structural support their providing is no longer necessary. Once implanted, this new class of biomaterials is expected to support the healing process of a diseased tissue or organ while degrading at a potentially adjustable degradation rate. We proposed recently that studying gene regulation of cells or a tissue in presence of DMM could provide interesting insights about their response [14]. In addition to help predict cell behaviour in the presence of degradation products, it would be possible to describe the mechanisms behind this response. In addition, determining a gene profile of a cell response towards DMMs could help identify potential gene products that could serve as biomarkers. The caveolin gene family seems to have the potential to become such biomarker. It shows a strong and reproducible down-regulation in presence of metals both at the level of gene expression and protein content. It is thus possible to envision studying tissues after implantation and evaluate caveolins expression in animal models after stent implantation.

Cav1 expression is regulated transcriptionally or post-translationally. The cav1 promoter region includes three G+C-rich sites which could act as sterol regulatory elements. Additionally, the promoter region contains a CAAT sequence and a Sp1 consensus sequence [15]. SRE-like elements are involved in the response to stimuli, such as low-density lipoprotein-free cholesterol (LDL-FC) [16,17]. Other transcription factors reported
to regulate cav1 expression include the forkhead (FKHR) family of transcription factors, FOXO3a [18], c-myc [16, 17] and NF-κB [19].

Several studies have implicated caveolins during the cellular response to a toxic stress. Endothelial cell caveolin-1 expression is down-regulated after exposure to nanoparticles. Since caveolin controls endothelial nitric oxide synthase, a decrease in Cav1 expression could lead to increase NO production and toxicity for the vascular wall [20]. Caveolin-1 levels are also reduced in endothelial cells exposed to cadmium [21]. On the other hand, treatment of cancer cells with cytotoxic agents (antineoplastic) usually increases caveolin expression [22-24]. Expression of caveolin is down-regulated in many cancer cells lines [25] while highly differentiated cells usually express high levels [26, 27]. In this study, we exposed fibroblasts and smooth muscle cells which have high levels of caveolins to DMM at moderately toxic concentrations and observed a loss of caveolins. Since caveolins are the principal structural protein of caveolae which both acts on plasma transport and signaling [26, 27], this down-regulation may be a way for the cells to protect themselves from the soluble metal oxides or metal ions present in the extracellular space by blocking the endocytosis capacity of caveolae.

Interestingly, the expression of cav-3 was also down regulated following metal powder and metal salt exposures, the same pattern as cav-1 showed. Although the role of cav-3 in signal transduction pathways towards cell survival is not well investigated, it has been reported to play a role in the phenotype and the death of cultured muscle cells [28].

In this study, the disruption of caveolin expression was closely related to the presence of metallic elements in the cell culture medium. Excessive presence of metallic elements
leads to a high oxidative stress for the cell and discarding caveolae may help the cell to preserve its integrity.

The implantation of a vascular stent is usually made in a region of an artery where atherosclerotic lesions are present. Restenosis is major problem related to stent implantation and this phenomenon is characterized by smooth muscle cell proliferation and increased artery wall fibrosis. The current use of drug-coated stent is aimed at avoiding this unwanted reaction to the stent interaction to the artery wall. Our present work here supports that the degradation of a stent made from DMM could lead to a regional cell proliferation inhibition which could also helped to slow the restenosis process. It will be interesting in the future to test if the effects of DMM on a real artery lead to caveolin down-regulation, a sign of reduced cellular proliferation.

**Conclusion**

The mRNA expression study showed that cav-1 was constantly down regulated in the presence of metallic elements both in the form of powders and salts. The protein expression level also showed a similar pattern where up to 48-hour incubation period, cav-1 was still down-regulated regardless the metal source. Interestingly, cav-3 showed the same expression pattern to that of cav-1, suggesting a general response of caveolin protein family towards oxidative stress generated by the presence of metallic elements within the cell culture medium. This finding suggests the potential of caveolin protein family as a biomarker for biocompatibility test of DMMs.
Acknowledgements

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References


Figure legends

Figure 1. Caveolin-1 (Cav1) mRNA levels in 3T3 fibroblasts after a 24- or a 48-hour exposure to powdered alloy, Mn, and Fe. The maximum diameter size of powder was 75 µm and the powder was added into the 3 µm tissue culture insert. Fibroblasts were pre-cultured for an overnight incubation period prior to metal exposures. Results are expressed as mean ± SEM (n=6) relative to mRNA levels measured in control (arbitrarily fixed at 1) *: p<0.001 vs. control.

Figure 2. Caveolin-1 (Cav1) protein content in 3T3 fibroblasts after a 24- or a 48-hour exposure to powdered alloy, Mn, and Fe. Results are expressed as mean ± SEM (n=6) relative to protein content measured in control (arbitrarily fixed at 1) *: p<0.001 vs. control. A view of the representative results from the caveolin-1 immunoblot for each treatment groups is illustrated at the bottom of the graph. The 22kDa unique band can be visualized.

Figure 3. Fibroblast cell count after a 24-hour exposure time with either increasing concentrations of FeCl₂ or MnCl₂. Fibroblasts were pre-cultured for an overnight incubation period prior to metal salt exposures. Cells were counted under light microscope using haemocytometer. Results are expressed as mean ± SEM (n=6).

Figure 4. Caveolin-1 (Cav1) mRNA levels in 3T3 fibroblasts after a 24- or a 48-hour exposure to FeCl₂ (0.15 mg/ml) or MnCl₂ (0.025 mg/ml). Fibroblasts were pre-cultured for an overnight incubation period prior to metal exposures. Results are expressed as mean ± SEM (n=6) relative to mRNA levels measured in control (arbitrarily fixed at 1) *: p<0.001 vs. control.
Figure 5. Caveolin-1 (Cav1) protein content in 3T3 fibroblasts after a 24- or a 48-hour exposure to FeCl$_2$ (0.15 mg/ml) or MnCl$_2$ (0.025 mg/ml). Fibroblasts were pre-cultured for an overnight incubation period prior to metal exposures. Results are expressed as mean ± SEM (n=6) relative to protein content measured in control (arbitrarily fixed at 1) *: p<0.001 vs. control. A view of the representative results from the caveolin-1 immunoblot for each treatment groups is illustrated at the bottom of the graph.

Figure 6. Caveolin-3 (Cav3) protein content in A10 smooth muscle cell line after a 24- or a 48-hour exposure to powdered alloy, Mn, and Fe. The maximum diameter size of powder was 75 µm and the powder was added into the 3 µm tissue culture insert. Cells were pre-cultured for an overnight incubation period prior to metal exposures. Top panels: Determination of cytostatic concentration of metal powders on A10 cells. Iron powder did not inhibit A10 cell proliferation and was used at the same concentration than the alloy. Bottom panel: Caveolin 3 protein content of A10 exposed to 4 mg/ml of the alloy and iron powder and 0.25mg/ml of manganese powder. Results are expressed as mean ± SEM (n=6) relative to protein content measured in control (arbitrarily fixed at 1) *: p<0.001 vs. control.

Figure 7. Caveolin-3 (Cav3) protein content in A10 smooth muscle cell line after a 24- or a 48-hour exposure to FeCl$_2$ (0.1mg/ml) or MnCl$_2$ (0.05mg/ml). Results are expressed as mean ± SEM (n=6) relative to protein content measured in control (arbitrarily fixed at 1) *: p<0.001 vs. control. A view of the representative results from the caveolin-3 immunoblot for each treatment groups is illustrated at the bottom of the graph.
Figure 8. Smooth muscle actin alpha (α-SMA) protein content in A10 smooth muscle cell line after a 48-hour exposure to FeCl₂ (0.1mg/ml) or MnCl₂ (0.05mg/ml). Results are expressed as mean ± SEM (n=6) relative to protein content measured in control (arbitrarily fixed at 1) *: p<0.001 vs. control. A view of the representative results from the alpha-smooth muscle actin immunoblot for each treatment groups is illustrated at the bottom of the graph.
Figure 2
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Figure 4

The graph shows the expression levels of Cav1 mRNA over time in different conditions. The x-axis represents time in hours (24h and 48h), and the y-axis represents Cav1 mRNA levels. There are three conditions: Control, FeCl2, and MnCl2. The bars with error bars indicate the mean mRNA levels, with asterisks (*) indicating statistically significant differences compared to the control group.

- At 24h:
  - Control: No significant change.
  - FeCl2: Decrease (significant).
  - MnCl2: Decrease (significant).

- At 48h:
  - Control: No significant change.
  - FeCl2: Further decrease (significant).
  - MnCl2: Further decrease (significant).
Figure 5

The bar chart shows the Cav1 protein content for Control, FeCl$_2$, and MnCl$_2$ treatments at 24h and 48h. The Western blot images below confirm the protein expression over time.

* indicates statistical significance.
Figure 7

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[Graph showing Cav3 protein content over time with Control, FeCl2, and MnCl2 conditions at 24h and 48h.]

[Western blot images for Cav3 and GAPDH at 24h and 48h, with asterisks indicating significant differences.]
Figure 8

The graph shows the α-SMA protein content for different conditions:

- Control
- FeCl₂
- MnCl₂

The bars for FeCl₂ and MnCl₂ are higher than the Control, indicating an increase in α-SMA protein content.

The Western blots below confirm these findings:

- α-SMA
- GAPDH