Xanthohumol inhibits growth and vascularization of developing endometriotic lesions


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BACKGROUND: Xanthohumol is a prenylated flavonoid isolated from hops, which is known to act as a pleiotropic cancer chemopreventive agent owing to its anti-proliferative, anti-inflammatory and anti-angiogenic properties. In the present study, we analyzed, for the first time, whether this dietary compound may also be used for the treatment of endometriosis.

METHODS: Peritoneal and mesenteric endometriotic lesions were surgically induced in BALB/c mice by uterine tissue transplantation into the abdominal cavity. The animals were treated daily with 100 µM xanthohumol (n = 8) or vehicle (control, n = 8) via the drinking water, starting 3 days before tissue transplantations. Lesion growth, cyst formation and vascularization were subsequently analyzed by means of high-resolution ultrasound imaging (at Day 0 and then once per week for 28 days), caliper measurements, western blotting, histology and immunohistochemistry over 4 weeks.

RESULTS: In the treatment and control groups, uterine grafts developed typical endometriotic lesions with cyst-like dilated glands surrounded by a vascularized endometrial stroma. However, xanthohumol efficiently decreased the size of these lesions at Day 28, independent of their localization within the peritoneal cavity, compared with control (peritoneal: P = 0.041; mesenteric: P = 0.038). This was associated with a reduced level of phosphoinositide 3-kinase protein. Moreover, vascularization of xanthohumol-treated lesions was suppressed, as indicated by a significantly lower microvessel density at Day 28 when compared with vehicle-treated controls (peritoneal: P = 0.026; mesenteric: P = 0.004). Additional analyses revealed that treatment with xanthohumol did not affect the histomorphology, proliferation and vascularization of the uterine horns and ovaries.

CONCLUSIONS: Taken together, these experimental findings suggest that xanthohumol inhibits the development of endometriotic lesions in mice without inducing serious side effects in the reproductive organs. Thus, xanthohumol represents a promising dietary phytochemical that, after further testing, may be considered for the use in the selective treatment of endometriotic lesions.

Key words: endometriosis / xanthohumol / angiogenesis / ultrasound / mice

Introduction

Endometriosis is defined as the presence of proliferating functional endometrium-like tissue outside the uterine cavity (Galle, 1989). It is estimated that 10–15% of women of reproductive age and up to 50% of all infertile women suffer from this frequent gynecological disease (Wheeler, 1989; Cramer and Missmer, 2002). Main symptoms are chronic pelvic pain, dysmenorrhea, dyspareunia and dysuria (Child and Tan, 2001).

According to the implantation theory of Sampson (1927), endometriotic lesions originate from endometrial fragments, which are shed into the peritoneal cavity during retrograde menstruation. Although these lesions are basically benign in nature, they share many similarities with developing tumors. In fact, both entities induce inflammatory processes, which involve the upregulation of nuclear factor kappa B (NFκB) signaling (González-Ramos et al., 2010; Ben-Neriah and Karin, 2011), the recruitment and activation of macrophages (Capobianco et al., 2011; Erreni et al., 2011) and the release of various pro-inflammatory cytokines (Harada et al., 2001; Grivennikov and Karin, 2011). Moreover, long-term survival and proliferation of both endometriotic lesions and tumors are crucially dependent on their adequate vascularization (Folkman, 1995; Groothuis et al., 2005; Laschke and Menger, 2007). Thus, treatment strategies in both diseases may include the application of anti-angiogenic compounds (Folkman, 1996; Taylor et al., 2009).
Xanthohumol is a prenylated flavonoid isolated from hops, which is known to act as a pleiotropic cancer chemopreventive agent, affecting a broad spectrum of cellular mechanisms (Gerhauser et al., 2002). It significantly reduces proliferation and induces apoptosis in different cancer cell lines (Pan et al., 2005; Drenzek et al., 2011). In addition, it exerts anti-inflammatory effects by suppressing NFkB signaling and the expression of inflammatory cytokines, such as interleukin-1β (Monteiro et al., 2008). Finally, xanthohumol has been shown to inhibit the secretion of vascular endothelial growth factor (VEGF) in acute and chronic myelogenous leukemia cell lines in vitro (Dell’Eva et al., 2007) and the development of new blood vessels in human breast cancer xenografts in vivo (Monteiro et al., 2008). This indicates that xanthohumol also effectively targets the process of angiogenesis.

Therefore, the aim of the present study was to analyze for the first time the effect of xanthohumol in endometriosis. For this purpose, we surgically induced endometriotic lesions in the peritoneal cavity of xanthohumol-treated and vehicle-treated mice and analyzed their size, proliferation and vascularization by means of non-invasive high-resolution ultrasound imaging, western blotting and immunohistochemical techniques (Körbel et al., 2010).

**Materials and Methods**

**Animals**

For the experiments, we used 10- to 14-week-old female BALB/c mice with a body weight of 18–20 g. The mice were housed eight per cage in a temperature-controlled environment under a 12 h/12 h light-dark cycle and had free access to the drinking solution and standard pellet food (Altromin, Lage, Germany). All experiments were approved by the local governmental animal care committee and were conducted in accordance with the German legislation on protection of animals and the NIH Guidelines for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, DC, USA).

The estrous stage of the animals was determined by vaginal lavage. For this purpose, 15 μl of 0.9% saline was carefully pipetted into the vagina. The suspension was then transferred on a glass slide and examined under a phase contrast microscope (CH-2; Olympus, Hamburg, Germany). To exclude discrepancies between individual animals related to differences in steroid hormone synthesis, only animals in the stage of estrous were used for the experiments.

**Model of intraperitoneal endometriosis**

Intraperitoneal endometriotic lesions were surgically induced by suturing uterine tissue samples to the abdominal wall, as described previously (Laschke et al., 2010). Donor mice were anesthetized by i.p. injection of ketamine (75 mg/kg body weight; Pharmacia GmbH, Erlangen, Germany) and xylazine 2% (Rompun, 15 mg/kg body weight; Bayer, Leverkusen, Germany). Both uterine horns were removed and transferred to a Petri dish containing 37°C warm Dulbecco’s modified Eagle medium (10% fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin; PAA, Colbe, Germany). The uterine horns were opened longitudinally with microscissors under a stereo-microscope (M651; Leica Microsystems GmbH, Wetzlar, Germany) and 2-mm tissue samples were removed using a dermal biopsy punch (Zeefel Laboratorium GmbH, Offenbach am Main, Germany) (Fig. 1A). Then, the tissue samples were fixed with a 6-0 prolene suture (Ethicon Products, Norderstedt, Germany) to the right and left abdominal wall (peritoneal lesions; n = 2) and to the intestinal mesentery (mesenteric lesions; n = 2) of the anesthetized recipient animals through a midline incision (Fig. 1B and C). Finally, the laparotomy was closed with running 6-0 prolene muscle and skin sutures. The development of peritoneal endometriotic lesions was analyzed by means of repetitive high-resolution ultrasound imaging throughout an observation period of 28 days.

**High-resolution ultrasound image acquisition and analysis**

Mice were anesthetized with 2% isoflurane in oxygen and fixed in supine position on a heated stage with electrocardiography electrodes and heart rate display (THM100; Indus Instruments, Houston, TX, USA). After chemical depilation (Nair hair removal lotion; Church & Dwight Canada Corp., Mississauga, ON, Canada) of the abdomen to prevent air trapped in the fur from interfering with ultrasound coupling into the animal, ultrasound coupling gel (Aquasonic 100; Parker, NJ, USA) was applied to the skin.

Ultrasound imaging of peritoneal lesions was performed with the Vevo 770TM high-resolution in vivo micro-imaging system (VisualSonics, Toronto, ON, Canada) and a real-time microvisualisation (RMV™) 704 Scanhead (VisualSonics) with a center frequency of 40 MHz and a focal depth of 6 mm (Laschke et al., 2010). The ultrasound images were analyzed by means of a three-dimensional reconstruction and analysis software licensed to VisualSonics for distribution with the Vevo 770™ high-resolution imaging system. The analyses included the determination of the overall volume of endometriotic lesion; their stromal tissue and cysts (in mm³) were determined by manual image segmentation. For this purpose, boundaries of endometriotic lesions and their cysts were manually outlined in parallel slices, separated by a step size of 200 μm.

**Figure 1** (A–C) Surgical induction of intraperitoneal endometriosis in the mouse model. For this purpose, a uterine tissue sample (arrow) is isolated from the longitudinally opened uterine horn of a BALB/c mouse by a 2-mm dermal biopsy punch (A) and sutured to the peritoneal wall of a syngeneic recipient mouse (B). Typical appearance of the tissue sample directly after fixation (C). Scale bars (A and B) = 2.5 mm; (C) = 1.25 mm.
in the three-dimensional ultrasound images. Based on the outlined areas, volumes were subsequently computed by the VisualSonics software. Moreover, we calculated the growth of lesions and stromal tissue (in % of the initial lesion and stromal tissue size) and we assessed the fraction of cyst-containing lesions (in % of all analyzed lesions).

After the last ultrasound imaging at Day 28, the animals were anesthetized by i.p. injection of ketamine and 2% xylazine and carefully laparotomized under a stereo-microscope to measure the largest and perpendicularly aligned smallest diameters of peritoneal and mesenteric endometriotic lesions by means of a digital caliper. The lesion sizes were then calculated with the formula $D_1 \times D_2 \times \pi/4$ (Becker et al., 2008). Subsequently, the animals were killed with an overdose of pentobarbital and the lesions as well as both uterine horns and ovaries were excised for further histological and immunohistochemical analyses.

**Histology and immunohistochemistry**

Formalin-fixed specimens of endometriotic lesions, uterine horns and ovaries were embedded in paraffin. Sections of 3-$\mu$m thickness were cut and stained with hematoxylin and eosin according to standard procedures.

Proliferating and apoptotic cells within the tissue samples were detected immunohistochemically using a mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (1:200; Dako Cytomation, Hamburg, Germany) and a rabbit polyclonal anti-cleaved caspase-3 antibody (1:100; Cell Signaling Technology, Boston, MA, USA) as primary antibodies. Subsequently, the tissue sections were incubated with avidin–peroxidase (1:50; Sigma-Aldrich, Taufkirchen, Germany) and the corresponding secondary antibodies. 3,3′-diaminobenzidine tetrahydrochloride was used as chromogen, counterstained with 1% methyl green. Numbers of PCNA- and cleaved caspase-3-positive cells (in % of all cells) were assessed within three high power fields of six peritoneal and six mesenteric lesions per group using a BX60 microscope (Olympus).

For immunofluorescent microscopic detection of microvessels, sections were stained with a monoclonal rat anti-mouse antibody against the endothelial cell marker CD31 (1:30; Dianova, Hamburg, Germany) and a rabbit polyclonal anti-cleaved caspase-3 antibody (1:100; Cell Signaling Technology, Boston, MA, USA) served as secondary antibodies. Cell nuclei were stained with Hoechst (1:500; Sigma-Aldrich). The microvessel density (mm$^{-2}$) of six peritoneal and six mesenteric lesions per group was measured using a BZ-8000 microscope (Keyence, Osaka, Japan). For the additional investigation of proliferation and apoptosis of the microvascular endothelium in the analyzed lesions, sections were stained with a monoclonal rat anti-mouse antibody against CD31 (1:30; Dianova) and a monoclonal mouse antibody against PCNA (1:200; Dako Cytomation) or a rabbit polyclonal antibody against cleaved caspase-3 (1:100; Cell Signaling Technology). A goat anti-rat AlexaFluor 488 antibody (1:200; Invitrogen, Eugene, USA) and a goat anti-mouse IgG Cy3 antibody (1:200; Jackson ImmunoResearch Europe Ltd., Suffolk, UK) or a goat anti-rabbit IgG Cy3 antibody (1:200; Jackson ImmunoResearch Europe Ltd) served as secondary antibodies. Cell nuclei were stained with Hoechst (1:500; Sigma-Aldrich). The fraction of PCNA/CD31-positive and cleaved caspase-3/CD31-positive endothelial cells (%) was measured using the BZ-8000 microscope (Keyence).

**Western blot analysis**

To investigate the effect of xanthohumol on kinase expression in our endometriosis model, endometriotic lesions were surgically induced in additional mice, as described above. After 28 days, the tissue was removed and stored in liquid nitrogen for western blot analysis of mitogen-activated protein (MAP) kinase (pErk1/2) and phosphoinositide 3-kinase (PI3-K) expression. Both kinases have previously been shown to be involved in the proliferation of endometriotic cells (Ngô et al., 2010; Zhang et al., 2010).

For extraction of the whole protein fraction, frozen tissue samples were homogenized in lysis buffer [10 mM Tris pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 0.5% Triton-X 100, 0.02% NaN$_3$, 0.2 mM phenylmethylsulfonyl fluoride and Protease-Inhibitor-Cocktail (1:100 v/v; Sigma-Aldrich)], incubated for 30 min on ice and centrifuged for 30 min at 16 000g (4°C). The supernatant was saved as whole protein fraction. Protein concentrations were determined using the Lowry assay with bovine serum albumin as standard. Thirty micrograms protein/lane were separated discontinuously on 10% sodium dodecylsulfate–polyacrylamide gels and transferred to a polyvinylidenefluoride membrane (BioRad, Munich, Germany). After blockage of non-specific binding sites, membranes were incubated for 4 h with a monoclonal mouse anti-pErk1/2 antibody (1:300; Abcam, Cambridge, UK) or with a monoclonal mouse anti-PI3-K antibody (1:300; BD Biosciences, Heidelberg, Germany) followed by the corresponding horse-radish peroxidase-conjugated secondary antibodies (1:5000; GE Healthcare, Freiburg, Germany). Protein was visualized using luminal-enhanced chemiluminescence and exposure of membranes to blue light-sensitive autoradiography film (Hyperfilm ECL, GE Healthcare). Signals were assessed densitometrically (Geldoc, Quantity one software, BioRad) and normalized to β-actin signals (monoclonal mouse anti-β-actin antibody, 1:500; Santa Cruz Biotechnology, Heidelberg, Germany) to correct for unequal loading.

**Experimental protocol**

The mice were divided into two groups (each $n = 8$) receiving either 100 μM xanthohumol (kindly provided by Hopsteiner, Mainburg, Germany) or 0.1% ethanol (vehicle; Sigma-Aldrich) via the drinking water. In a previous study, this dosage of xanthohumol effectively inhibited the vascularization of breast cancer xenografts (Monteiro et al., 2008). To avoid degradation of xanthohumol, the drinking solutions were kept in dark bottles and renewed every day. The treatment started 3 days before transplantation of uterine tissue samples into the abdominal cavity. Ultrasound image analyses of the grafts were performed directly after tissue transplantation (d0) as well as at Day 7, 14, 21 and 28. At the end of the experiments, the size of developing endometriotic lesions was measured by means of a digital caliper. Subsequently, the lesions, uterine horns and ovaries were excised for further histological and immunohistochemical analyses.

In a subset of experiments, six peritoneal and three mesenteric lesions per animal were surgically induced in additional three mice per group and harvested at Day 28 for western blot analyses. For this purpose, the lesions had to be pooled, because individual lesion sizes were too small to guarantee adequate measurements of protein expression.

**Statistics**

Data were first analyzed for normal distribution and equal variance. In case of parametric data, differences between the two experimental groups were assessed by the unpaired Student’s t-test. In case of non-parametric data, differences between the two experimental groups were assessed by the Mann–Whitney rank sum test. To test for time effects within each experimental group, analysis of variance for repeated measurements was applied. This was followed by the Dunnet post hoc test (SigmaStat; Jandel Corporation, San Rafael, CA, USA). All data are given as mean ± SEM. A value of $P < 0.05$ was considered as statistically significant.
**Results**

**General remarks**

Xanthohumol-treated and control mice drank 4.6 ± 0.2 and 5.2 ± 0.2 ml per day (P < 0.05), respectively. Accordingly, the concentration of 100 μM xanthohumol given in the drinking water corresponded to a daily xanthohumol dose of about 8 mg/kg body weight. The mice tolerated the daily treatment with xanthohumol well, as indicated by normal feeding, cleaning and sleeping habits, which did not differ from those of vehicle-treated control animals. Accordingly, the mice exhibited a physiological gain in weight throughout the 4-week observation period.

**Xanthohumol action on growth and cyst formation of endometriotic lesions**

In the present study, endometriotic lesions were surgically induced by suturing uterine tissue samples to the lateral abdominal wall or the intestinal mesentery. In contrast to the mesenteric lesions, endometriotic lesions of the abdominal wall were not affected by respiratory movements or peristalsis of the intestine. Thus, peritoneal lesions were used for detailed repetitive analyses of growth and cyst formation by means of high-resolution ultrasound imaging (Fig. 2).

Quantitative analysis of ultrasound images demonstrated that the peritoneal lesions of xanthohumol- and vehicle-treated mice exhibited a comparable initial volume of about 1.0 mm³ (Fig. 2C). During the further time course of the experiment, the lesion volume of control animals progressively increased (Fig. 2C and D). This was caused by the growth of stromal tissue and the volume increase of developing cysts within the lesions (Fig. 2E–G). In contrast, xanthohumol treatment markedly inhibited the growth of endometriotic lesions, as indicated by a significantly lower lesion and stromal tissue volume between Day 14 and 28 when compared with controls (Fig. 2A–F). Accordingly, these lesions exhibited also a markedly decreased rate of lesion and stromal tissue growth (Fig. 2D and F). In addition, the cyst volume of lesions in xanthohumol-treated animals was lower in comparison with controls (Fig. 2G), although both groups exhibited a comparable fraction of cyst-containing lesions between Day 14 and 28 (Fig. 2H).

In line with these results, histological analyses at Day 28 revealed that the treatment with xanthohumol suppressed the development of peritoneal and mesenteric endometriotic lesions (Fig. 3A–D). Accordingly, the sizes of peritoneal and mesenteric lesions as assessed by caliper measurements were significantly lower in xanthohumol-treated mice when compared to those in controls (Fig. 3E).

**Xanthohumol action on vascularization of endometriotic lesions**

Immunohistochemical detection of CD31-positive microvessels showed that xanthohumol effectively inhibited the formation of new blood vessels in developing endometriotic lesions (Fig. 4A–D). In fact, both peritoneal and mesenteric lesions of xanthohumol-treated animals exhibited a significantly lower microvessel density at Day 28 when compared with vehicle-treated controls (Fig. 4E). More detailed immunohistochemical analyses revealed that this was associated with a reduced proliferating activity of the microvascular endothelium in xanthohumol-treated lesions (Fig. 5), whereas xanthohumol treatment did not induce apoptotic cell death of endothelial cells (data not shown).

**Xanthohumol action on apoptosis and cell proliferation in endometriotic lesions**

Immunohistochemical staining of cleaved caspase-3 showed that peritoneal and mesenteric lesions of xanthohumol-treated and vehicle-treated control animals did not contain any apoptotic cells at Day 28 after surgical induction. Lesions of the control group exhibited ~20% of PCNA-positive cells (Fig. 6A, C and E). This high proliferating activity was significantly reduced in lesions of xanthohumol-treated animals (Fig. 6B, D and E).

**Xanthohumol action on kinases in endometriotic lesions**

To clarify, whether the observed anti-proliferative effect of xanthohumol was induced by a decreased kinase expression in endometriotic lesions, we additionally performed western blot analyses. By this, we could demonstrate that treatment with xanthohumol did not affect the level of pErk1/2 protein (Fig. 7). However, lesions of xanthohumol-treated animals exhibited a significantly reduced level of PI3-K protein when compared with those of vehicle-treated controls (Fig. 7).

**Xanthohumol action on the female reproductive organs**

To detect potential side effects of xanthohumol treatment on the female reproductive organs, we performed histological and immunohistochemical analyses of the uterine horns and ovaries of animals at Day 28 after surgical induction of endometriotic lesions. However, we did not detect any differences between xanthohumol-treated and vehicle-treated control animals. In both groups, the uterine horns and ovaries presented with a normal histomorphology (Fig. 8A and B; 9A and B). In addition, they did not exhibit any functional differences in terms of vascularization and cell proliferation (Fig. 8 and 9C–F).

**Discussion**

The hop compound xanthohumol is a typical pleiotropic agent, which has been shown to exert anti-proliferative, anti-inflammatory and anti-angiogenic effects in a wide variety of cancer cell lines (Miranda et al., 1999; Dell’Eva et al., 2007; Monteiro et al., 2008). Accordingly, xanthohumol has been suggested to be a promising dietary phytochemical for cancer prevention and therapy (Stevens and Page, 2004). Herein, we now demonstrate for the first time that xanthohumol may also be used for the treatment of endometriosis. In fact, we found that xanthohumol effectively suppressed the development of endometriotic lesions, which were surgically induced in the peritoneal cavity of BALB/c mice.

In our study, xanthohumol was administered in a concentration of 100 μM (~8 mg/kg body weight) via the drinking water. This is a rather low dose, considering the fact that the compound’s bioavailability is extremely poor, possibly related to extensive intestinal metabolism by gut microorganisms (Avula et al., 2004; Stevens and Page,
However, we decided to use this dose and route of application, because in a recent study of Monteiro et al. (2008) this treatment regime significantly inhibited the vascularization of developing tumors in mice, which was associated with central tumor cell necrosis. These findings and our own results indicate that even low xanthohumol concentrations can exert relevant beneficial effects under different pathological conditions, supporting the concept of using xanthohumol as a dietary supplement for the therapy of cancer or endometriosis.

**Figure 2** High-resolution ultrasound imaging of developing endometriotic lesions (borders marked by red broken line, cysts marked by yellow broken line) at the abdominal wall of BALB/c mice 28 days after treatment with 0.1% ethanol (A, control) or 100 μM xanthohumol (B) via the drinking water. Scale bars: 1 mm. (C–H) Overall lesion volume (C, mm³), lesion growth (D, %), stromal tissue volume (E, mm³), stromal tissue growth (F, %), cyst volume (G, mm³) and fraction of cyst-containing lesions (H, %) of BALB/c mice, which were treated with 0.1% ethanol (control; white bars; n = 8) or 100 μM xanthohumol (black bars; n = 8) via the drinking water. Mean ± SEM; *P < 0.05 versus control; †P < 0.05 versus Day 0.
For this purpose, the compound may be extracted and purified from genetically transformed hops with high xanthohumol content (Gatica-Arias et al., 2011).

In line with former studies (Monteiro et al., 2008; Negrão et al., 2012), we found that xanthohumol inhibits the process of angiogenesis, resulting in a markedly decreased vascularization of endometriotic lesions. This anti-angiogenic action of xanthohumol is most probably mediated by multiple mechanisms. Of interest, we could demonstrate that xanthohumol exerts an anti-proliferative effect on microvessels within endometriotic lesions without inducing apoptosis of endothelial cells, which may be explained by the direct suppression of VEGF signaling (Dell’Eva et al., 2007; Negrão et al., 2012). On the other hand, xanthohumol is able to scavenge reactive oxygen species (ROS), including hydroxyl- and peroxy radicals (Gerhauser et al., 2002). ROS, in turn, have been shown to be up-regulated in endometriosis (Ngô et al., 2009). Moreover, oxidative stress promotes VEGF expression in endometrial cells (Park et al., 2006; Taylor et al., 2009). Thus, although not analyzed further, the anti-oxidative effect of xanthohumol may have additionally contributed to the reduced lesion vascularization in our endometriosis model.

Considering the fact that the survival and proliferation of endometriotic lesions is crucially dependent on a sufficient blood supply (Groothuis et al., 2005; Laschke and Menger, 2007), the observed anti-angiogenic action of xanthohumol represents an obvious explanation for the reduced number of PCNA-positive cells in xanthohumol...

Figure 3 (A–D) Hematoxylin–eosin stained cross sections of endometriotic lesions (borders marked by broken line) at Day 28 after surgical induction by fixation of uterine tissue samples to the peritoneal wall (A and B) or the intestinal mesentery (C and D) of BALB/c mice, which were treated with 0.1% ethanol (control; A and C) or 100 μM xanthohumol (B and D) via the drinking water. Note that the lesions of xanthohumol-treated mice exhibit a markedly reduced size when compared with controls. Scale bars: 800 μm. (E) Size (mm²) of peritoneal and mesenteric endometriotic lesions of control (white bars; n = 8) and xanthohumol-treated (black bars; n = 8) BALB/c mice, as assessed by caliper measurement. Mean ± SEM; *P < 0.05 versus control.

Figure 4 Immunofluorescent cross sections of endometriotic lesions at Day 28 after surgical induction by fixation of uterine tissue samples to the peritoneal wall (A and B) or the intestinal mesentery (C and D) of BALB/c mice, which were treated with 0.1% ethanol (control; A and C) or 100 μM xanthohumol (B and D) via the drinking water. Sections were stained with Hoechst to identify cell nuclei (blue) and an antibody against CD31 for the detection of the microvascular endothelium (red). Note that the lesions of xanthohumol-treated mice exhibit a markedly lower microvessel density. Scale bars: 50 μm. (E) Microvessel density (mm⁻²) of peritoneal and mesenteric endometriotic lesions of control (white bars; n = 6) and xanthohumol-treated (black bars; n = 6) BALB/c mice. Mean ± SEM; *P < 0.05 versus control.
treated lesions. However, treatment with the hop compound may have also affected hormone-driven growth of the lesions owing to its anti-estrogenic activity (Gerhauser et al., 2002). Moreover, xanthohumol directly inhibits cell proliferation, as previously demonstrated for different cancer cell lines (Colgate et al., 2007; Drenzek et al., 2011). Our western blot results indicate that this may be mediated by the suppression of PI3-K signaling in endometriotic lesions, whereas xanthohumol treatment does not affect the expression of pErk1/2.

Because under clinical conditions the distribution of endometriotic lesions inside the peritoneal cavity shows a high variability, we tested in the present study the effect of xanthohumol on lesions of different locations, i.e. the lateral abdominal wall and the mesentery. In contrast to mesenteric lesions, lesions of the lateral abdominal wall could easily be analyzed by means of high-resolution ultrasound imaging, because they were not affected by respiratory movements or peristalsis of the intestine. The ultrasound analyses revealed that xanthohumol treatment did not only inhibit the proliferation of the stromal tissue fraction, but also resulted in smaller cyst volumes. Thus, xanthohumol may influence the secretory activity of the glandular epithelium. In addition, we found in line with former studies (Stoeckemann et al., 1995) that the average size of mesenteric

**Figure 5** Immunofluorescent cross sections of endometriotic lesions at Day 28 after surgical induction by fixation of uterine tissue samples to the peritoneal wall (A and B) or the intestinal mesentery (C and D) of BALB/c mice, which were treated with 0.1% ethanol (control; A and C) or 100 µM xanthohumol (B and D) via the drinking water. Sections were stained with Hoechst to identify cell nuclei (blue), an antibody against CD31 for the detection of the microvascular endothelium (green) and an antibody against the proliferation marker PCNA (red). Note that the lesions of xanthohumol-treated mice exhibit a markedly reduced number of proliferating endothelial cells. Scale bars: 20 µm. (E) PCNA/CD31-positive cells (%) of peritoneal and mesenteric endometriotic lesions of control (white bars; n = 6) and xanthohumol-treated (black bars; n = 6) BALB/c mice. Mean ± SEM; *P < 0.05 versus control.

**Figure 6** Immunohistochemical cross sections of endometriotic lesions at Day 28 after surgical induction by fixation of uterine tissue samples to the peritoneal wall (A and B) or the intestinal mesentery (C and D) of BALB/c mice, which were treated with 0.1% ethanol (control; A and C) or 100 µM xanthohumol (B and D) via the drinking water. Sections were stained with an antibody against PCNA for the detection of proliferating cells. Note that the lesions of xanthohumol-treated mice exhibit a markedly reduced cell proliferation. Scale bars: 50 µm. (E) PCNA-positive cells (%) of peritoneal and mesenteric endometriotic lesions of control (white bars; n = 6) and xanthohumol-treated (black bars; n = 6) BALB/c mice. Mean ± SEM; *P < 0.05 versus control.
lesions at Day 28 was increased when compared with peritoneal lesions. This may be caused by the improved blood supply in the intestinal mesentery. However, the two lesion types did not show a consistent difference in the treatment response to xanthohumol. Therefore, we assume that the efficiency of xanthohumol treatment is not essentially dependent on the localization of endometriotic lesions inside the peritoneal cavity.

Besides the analysis of endometriotic lesions, we investigated also the effect of xanthohumol treatment on the uterus and the ovary in order to evaluate whether xanthohumol might induce severe side effects in the organs of the reproductive tract. This is not unlikely, because these organs are characterized by a high angiogenic and proliferative activity (Reynolds et al., 1992, 2002). Of interest, we found that the uterine horns and ovaries of both xanthohumol-treated and control animals presented with a normal histomorphology, microvessel density and cellular proliferating activity. These results are in line with a safety study of oral xanthohumol administration by Hussong et al. (2005), reporting that even higher doses of 100 mg/kg body weight xanthohumol did not cause any adverse effects on fertility and the development of offspring.

Figure 7  Protein levels [optical density (OD)*mm²] of MAP kinase (pErk1/2) and PI3-K in peritoneal and mesenteric endometriotic lesions of control (white bars; n = 3) and xanthohumol-treated (black bars; n = 3) BALB/c mice, as assessed by western blot analysis. Mean ± SEM; *P < 0.05 versus control.

Figure 8  Histological sections of uterine horns from BALB/c mice, which were treated for 28 days with 0.1% ethanol (control; A, C and E) or 100 μM xanthohumol (B, D and F) via the drinking water [A and B: hematoxylin–eosin staining; C and D: staining with Hoechst to identify cell nuclei (blue) and an antibody against CD31 for the detection of the microvascular endothelium (red); E and F: staining with an antibody against PCNA for the detection of proliferating cells]. Note that the uterine horns of both groups exhibit a normal histomorphology, microvessel density and cellular proliferating activity. Scale bars (A and B) = 500 μm; (C–F) = 170 μm.

Figure 9  Histological sections of ovaries from BALB/c mice, which were treated for 28 days with 0.1% ethanol (control; A, C and E) or 100 μM xanthohumol (B, D and F) via the drinking water [A and B: hematoxylin–eosin staining; C and D: staining with Hoechst to identify cell nuclei (blue) and an antibody against CD31 for the detection of the microvascular endothelium (red); E and F: staining with an antibody against PCNA for the detection of proliferating cells]. Note that the ovaries of both groups exhibit a normal histomorphology, microvessel density and cellular proliferating activity. Scale bars: (A and B) = 500 μm; (C–F) = 170 μm.
in rats. This suggests that xanthohumol may be useful for the selective treatment of endometriotic lesions, without affecting the reproductive organs.

Taken together, we could demonstrate for the first time that xanthohumol inhibits growth and vascularization of endometriotic lesions. Accordingly, this hop compound represents a promising dietary phytochemical, which, after further testing, may be useful in the future for the treatment of endometriosis.

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Authors’ roles

J.R.-A.: acquisition of data, analysis and interpretation of data; drafting the article; final approval of the article. C.K. and M.D.M.: interpretation of data; revising the article; final approval of the article. C.S.: acquisition of data, analysis and interpretation of data; final approval of the article. M.W.L.: supervision of experiments; interpretation of data; revising the article; final approval of the article.

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Conflict of interest

None declared.

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