Post-injury *ex vivo* model to investigate effects and toxicity of pharmacological treatment in rings of rabbit aortic vessels

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Summary
Animal experiments are widely accepted in arteriosclerosis research. The aim of the present study was to establish an organ culture model (rings of rabbit aortic vessels) to investigate inhibitory estrogen effects on post injury neointima formation in the vessel wall and to examine whether these effects are cytotoxic. Estrogens are used for secondary prevention of atherosclerosis in postmenopausal women (estrogen replacement therapy/ERT). Phytoestrogens as well as the ovarian 17β-estradiol have been demonstrated to inhibit proliferation and migration of vascular smooth muscle cells which are key events in atherogenesis and restenosis after coronary angioplasty.
In situ endothelial denudation of the thoracic and abdominal aorta was performed in female rabbits by a 3F Fogarty catheter. Segments of 5 mm were randomized in groups of n = 12 and held in culture. 17β-estradiol, Genistein and Daidzein were applicated in concentrations of 20 μM, 30 μM, and 40 μM. Groups without estrogen treatment served as controls. The segments were investigated after 21 days. Afterwards, 3 further groups (n = 12) were held with the lowest concentrations of 17β-estradiol or the two phytoestrogens having been evaluated to inhibit the neointima formation significantly. After 21 days of treatment these sections were held in medium only for another 7 days to proof whether these segments were still able to proliferate. A denuded control group was held in medium only over 28 days. Compared to controls, 30 μM 17β-estradiol, 20 μM Genistein, and 40 μM Daidzein inhibited neointima formation significantly over 21 days. After another 7 days of cultivation in medium only the amount of neointima formation was comparable to that of non-estrogen-treated controls after 21 days. We therefore suggest that the demonstrated inhibitory effect is not explained by toxicity. In conclusion, by the use of this organ culture model it was possible to demonstrate non-toxic post injury effects of different estrogens in the vasculature. Because 24 aortic segments could be taken from one aortic vessel, the number of animals that would have been necessary for an experiment (8 to 10 per group for statistical reasons) could be markedly reduced. The results are of clinical interest because phytoestrogens and 17β-estradiol may offer therapeutic options for patients after coronary angioplasty regarding the process of restenosis. Because phytoestrogens do not affect the reproductive system they can also be used in men.

Keywords: 3R, reduction, organ culture, aortic vessel, 17β-estradiol, phytoestrogens, toxicity

Zusammenfassung: Ex vivo Modell zur Untersuchung der Toxizität pharmakologischer Wirkungen auf die Verletzungsreaktion in Aortenringen von Kaninchen
Tierexperimente werden häufig in der Arterioskleroseforschung eingesetzt. Das Ziel der vorliegenden Studie war es, ein Organkulturmodell zu etablieren (Ringe aus der Kaninchenaorta), mit dem zum einen die Wirkung von Östrogenen auf die Verletzungsreaktion der Gefässwand überprüft werden kann; darüber hinaus, ob die gezeigte Wirkung auf toxischen Effekten beruht. Östrogene werden heute zur Sekundärprävention der koronaren Herzerkrankung bei postmenopausalen Frauen eingesetzt (Hormon Ersatztherapie). Es wurde bereits früher beschrieben, dass Phytoöstrogene und das ovarielle 17β-Östradiol die Migration und Proliferation glatter Muskelzellen in der Gefässwand hemmen können. Diese Mechanismen sind Schlüsselprozesse bei der Atherogenese und auch der Restenose nach einer Koronarangioplastie. Es wurde hier in situ eine Endotheldenudation der Aorta abdominalis und Aorta thoracalis mittels Ballonkatheter durchgeführt. Segmente von 5 mm Länge wurden in Gruppen zu n = 12 randomisiert und kultiviert. 17β-Östradiol, Genistein und Daidzein wurden in Konzentrationen von 20 μM, 30 μM und 40 μM hinzu titriert. Gruppen ohne Östrogenbehandlung dienten der Kontrolle. Die Segmente wurden nach 21 Tagen untersucht. Danach wurden drei weitere Gruppen mit denudierten Gefässsegmenten (n = 12) gebildet, die mit den jeweils niedrigsten Konzentrationen von 17β-Östradiol, Genistein und Daidzein behandelt wurden, die über 21 Tage die Neointimabildung signifikant gehemmt hatten. Nach 21 Tagen Behandlungsdauer wurde diese Segmente für weitere 7 Tage ohne die Östrogenbehandlung kultiviert um zu testen, ob die Gefässwände noch in der Lage waren zu proliferieren. Eine Gruppe mit 12 denudierten Gefässsegmenten wurde als Kontrolle für 28 Tage nur in Medium kultiviert. Im Vergleich zur Kontrollgruppe (21 Tage) waren 30 μM 17β-Östradiol, 20 μM Genistein und 40 μM Daidzein in der Lage, nach Gefässverletzung die Neointimabildung über 21 Tage signifikant zu hemmen. Nach weiteren 7 Tagen Kultivierung in Medium ohne Östrogenbehandlung entsprach der Grad der Neointimabildung dem bei der denudierten Kontrollgruppe über 21 Tage. Wir schliessen daraus, dass der gezeigte inhibitorische Effekt auf die Neointimabildung nicht durch toxische Wirkungen erklärt werden kann. Immunhistochemische Färbungen zeigten, dass die Wirkung eher durch eine Hemmung der Migration glatter Muskelzellen als durch Hemmung von deren Proliferation erklärt werden kann. Durch den Einsatz dieses Organkultur-

modells war es möglich, nicht-toxische Effekte verschiedener Östrogene auf die Verletzungsreaktion der Gefässwand zu zeigen. Weil 24 Aortensegmente aus dem Gefäss eines Tieres gewonnen werden konnten, wurde die Zahl der Versuchstiere im Vergleich zu einem Tierexperiment (aus statistischen Gründen 8–10 pro Gruppe) deutlich reduziert. Diese Ergebnisse haben auch klinische Bedeutung, denn Phytoöstrogene und 17β-Östradiol könnten einen Therapieansatz bei Patienten nach Koronarangioplastie zum Schutz vor einer Restenose bilden. Da Phytoöstrogene keine Wirkung auf das Reproduktionssystem haben, können sie auch bei Männern eingesetzt werden.

1 Introduction

Animal experiments are widely accepted in cardiovascular research and have been used frequently by our working group (Hanke et al., 1990; Hanke et al., 1996; Hanke et al., 1999; Finking et al., 2000). However, it can be helpful to investigate distinct pharmacological effects in a less complex model before creating a study with living creatures, with regard to ethical and economical questions. The aim of this present study was to establish an organ culture model (rings of rabbit aortic vessels) to investigate pharmacological effects in the vessel wall and to proof whether the effects are mediated by toxic mechanisms.

Estrogen replacement therapy (ERT) has been a widely accepted secondary preventive strategy against coronary artery disease in postmenopausal women since a number of cohort studies had found an association of reduced cardiovascular mortality with estrogen treatment (Sullivan et al., 1990; Grodstein et al., 1997). However, a randomized and double blinded study on combined estrogen/progesterone treatment in postmenopausal women with coronary artery disease did not find any benefit after a follow-up period of 4.1 years (Hulley et al., 1998). This may be due to the combined progesterone treatment which is necessary to reduce the risk of endometrial hyperplasia and endometrial cancer.

Key events in atherogenesis and in restenosis after vascular trauma are the migration and proliferation of medial vascular smooth muscle cells (Ross, 1985; Rosenfeld and Ross, 1990) and of adventitial myofibroblasts (Shi et al., 1996). Therefore, the effects of estrogens on these processes are under intensive investigation. In cell and organ culture experiments several authors were able to demonstrate inhibitory properties of 17β-estradiol on vascular smooth muscle cell proliferation (Vargas et al., 1993; Fischer-Dzoga et al., 1983; Voisard et al., 1995; Suzuki et al., 1996; Akishita et al., 1997). On the other hand, (Farhat et al., 1992) saw enhanced proliferation in vascular smooth muscle cells from rat pulmonary artery and in endothelium denuded rabbit pulmonary artery segments under 17β-estradiol treatment.

Animal experiments were designed to investigate whether 17β-estradiol's presumed antiproliferative properties could be of clinical relevance, i.e., by reducing post-injury neointima development which is in part a proliferative response (Ross, 1985; Rosenfeld et Ross, 1990; Shi et al., 1996). Indeed, several authors were able to demonstrate a reduction of post-injury neointima formation by 17β-estradiol treatment in carotid arteries from rats (Chen et al., 1996; Levine et al., 1996; Krasinski et al., 1997; Oparil et al., 1997; White et al., 1997) and mice (Sullivan et al., 1995; Iafrati et al., 1997) and in aortas from rabbits (Foegh et al., 1994). These findings do still need to be reproduced in humans.

Because nutrition plays an important role in the prevention of cardiovascular diseases (Simons, 1986; Pauletto et al., 1996; Barnes, 1998), destinct ingredients, i.e., phytoestrogens are supposed to influence the cardioprotective effect (Finking et al., 1998, 1999). Phytoestrogens could be extracted from plants, especially from soy beans (Coward et al., 1996; Reinli and Block, 1996). Their chemical structure is similar to that of endogenous estrogen and they interact with estrogen receptors in different tissue (Rosenblum et al., 1993; Whitten et al., 1992). Well-known phytoestrogens are Genistein and Daidzein (Rosenblum et al., 1993; Pelissero et al., 1991; Xu et al., 1994). Genistein is discussed to have antiproliferative properties regarding tumor growth (Fotsis et al., 1995) and atherogenesis (Raines and Ross, 1995; Anthony et al., 1998). Moreover, phytoestrogens have been shown to inhibit migration and proliferation as well as matrix synthesis of human vascular smooth muscle cells *in vitro* (Dubey et al., 1999).

Therefore, in the present experiment the phytoestrogens Genistein, its analogue Daidzein, and 17β-estradiol were tested in comparable concentrations regarding their influence on post-injury processes in the vasculature. While Genistein is well investigated as an inhibitor of the protein tyrosine kinases (Akiyama et al., 1987; Huang et al., 1992; Bischof et al., 1995) which could possibly explain some antiproliferative properties, its analogue Daidzein lacks this effect (Negrescu et al., 1995; Bischof et al., 1995; Jonas et al., 1995).

On the background of our experiences with experiments in rabbits the purpose of this *in vitro* study was to establish an *ex vivo* organ culture model (rabbit aorta). The aim was to find concentrations of 17β-estradiol and the two phytoestrogens that are able to decrease neointima formation over 21 days after vascular injury with a balloon catheter. In order to further demonstrate that the inhibitory effect was not a toxic one, the aortic segments were held in culture without estrogen or phytoestrogen treatment for another 7 days testing whether they were still able to proliferate and to build up a neointima. The experiments run over 21/28 days because it has been shown previously that post injury proliferation reaches a maximum after one to two weeks and matrix synthesis is completed four weeks after injury in rabbit aortic vessels (Hanke et al., 1990).

2 Methods

2.1 *In vitro* model

A total of 5 adult female New Zealand White (NZW) rabbits with 3 kg body weight (Tierforschungszentrum, Universität D-Ulm) were killed by shooting a bolt into the back of their heads and exsanguinated by cutting the carotid arteries. The abdomen was opened by a scalpel and the aortic vessel prepared by removal of the connective tissue. Endothelium denudation of the abdominal and thoracic aorta was then performed in all animals *in situ* with a 3F Fogarty catheter (Baxter Inc., D-Unterschleissheim) which was pushed into the vessel through an incision at the iliac bifurcation. After inflation with natrium chloride 0.9% the balloon was pulled through the whole vessel one times. The now denuded aortae were excised by saving the adventitial tissue, cut into 24 sections of 5 mm and randomised into 10 groups of 12 aortic rings each. One

group served as a control group and was held in medium containing 1% isopropanol (Roth, D-Karlsruhe) and 1% dimethyl sulphoxide (DMSO) (Sigma, D-Deisenhofen) because 17β-estradiol, Genistein and Daidzein were dissolved in isopropanol and DMSO of the same concentration. The other 9 groups were treated with 17β-estradiol, Genistein or Daidzein (Sigma, D-Deisenhofen) in concentrations of 20 µM, 30 µM or 40 µM.

All aortic rings were held separately in six-well plates for 21 days at 37°C with phenol red free Dulbecco's modified Eagle medium (DMEM) with Ham's F12 (mixed 1 plus 4; Gibco, Eggenstein, Germany), containing D-glucose (4.5 g/l), 15% fetal calf serum (fcs) (Bio Whittacker, D-Heidelberg) and 5 ml/l Penicillin-Streptomycin (Gibco, D-Eggenstein). The medium contained 1% isopropanol and 1% DMSO in all groups and was renewed together with the estrogens three times a week.

In a second step two further female rabbits were killed as described above, exsanguinated and their afterwards balloon injured aortae were cut in 5 mm sections building 4 groups with 12 sections each. Three groups were treated with the lowest concentrations of 17β-estradiol (30 µM), Genistein (20 µM), or Daidzein (40 µM) having been found to inhibit neointima formation significantly over 21 days. One group served as a denuded control. After 21 days of treatment the sections were rinsed with PBS buffer (Dulbecco's PBS, Gibco, D-Eggenstein), 500 ml PBS buffer containing 7,5 ml Hepes-buffer-solution (Hepes 1M; Sigma, D-Deisenhofen). Afterwards the sections were held in medium without 17β-estradiol/phytoestrogen treatment but with 20% fcs for another 7 days, while the medium was renewed three times.

2.2 Immunohistochemistry, morphometry, statistical evaluation

The sections were fixed in 4% formaline, embedded in paraffin, and serially cut (4 µm slices) until the maximal thickness of the neointima was reached. Elastica-van-Gieson's staining was performed for the morphometry of the neointima (software package from Bilaney Consulting Inc, D-Düsseldorf). The neointimal area was defined the area between lamina elastica interna and lumen. Histomorphometry was done in a blinded fashion. Haemalaun and Eosin staining was performed for the identification of cell nuclei and the neointimal cell count was done under a microscope (magnification 20 x–40 x).

To identify smooth muscle cells and myofibroblasts among medial and neointimal cells immunohistochemical staining (biotin avidin peroxidase method) was performed with a monoclonal antibody against α-actin (mouse-anti-human; Renner Inc, D-Darmstadt).

Proliferating cells were identified by staining for 5-Bromo-2'deoxy-Uridine (30.71 mg BrdU + 26.37 mg 2'Desoxycytidine; Sigma, D-Deisenhofen) which is incorporated into the nuclear DNA during the S-phase of the cell cycle and was administered to the medium in a 20 µM concentration 18 hours before the fixation of the organ sections. Staining of BrdU was performed by the biotin avidin peroxidase method using a monoclonal mouse antibody against BrdU (Biocell Consulting, D-Reinach).

Results are expressed as mean and standard deviation. The U-test (Mann-Whitney-Wilcoxon) was used to determine statistical significance at a level of $p<0.05$. The proliferative activity in the neointima is expressed as the percentage of BrdU-positive cells. The amount of α-actin positive cells was estimated in a semi-quantitative manor using a score: + = 0–25% positive cells; ++ = 26–50% positive cells; +++ = 51–75% positive cells; ++++ = 76–100% positive cells.

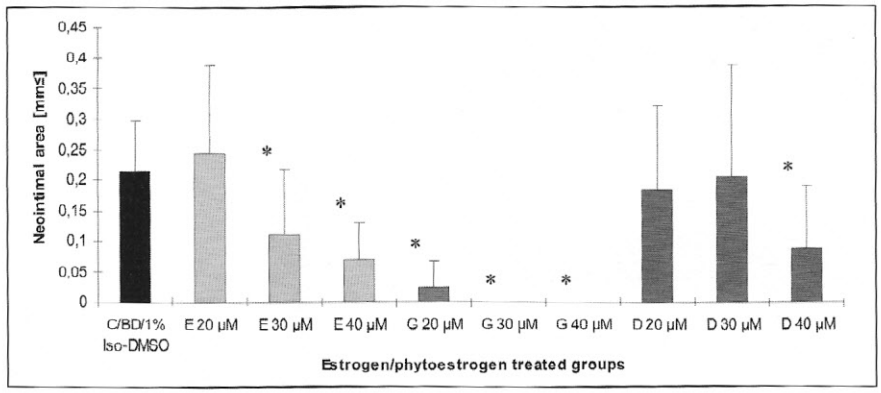

Figure 1: Inhibitory effect of 17β-estradiol (E), Genistein (G) and Daidzein (D) on post-injury neointima development in concentrations of 20/30/40 µM, compared to endothelium denuded (BD) controls (C). The medium of all groups contained 1% isopropanol (1% iso) and 1% DMSO. Compared with controls 17β-estradiol, Genistein and Daidzein (*) reduced neointima formation significantly ($p<0.05$) in a concentration dependent manor. Genistein had the strongest inhibitory effect.

3 Results

3.1 Neointimal plaque size

21 days of cultivation in medium with 1% isopropanol and 1% DMSO resulted in a measurable neointima formation in endothelium denuded female rabbit aortic sections (Fig. 1). 17β-estradiol, Genistein and Daidzein, compared with controls, were able to reduce the neointima formation significantly in a dose dependent manor (Fig. 1): 17β-estradiol in concentrations of 30 µM and 40 µM; Genistein in concentrations of 20 µM, 30 µM and 40 µM; Daidzein in concentrations of 40 µM. In comparable dosages Genistein had the strongest inhibiting effect.

The lowest concentrations of the three estrogens having been established to reduce neointima formation significantly over 21 days (30 µM 17b-estradiol, 20 µM Genistein, 40 µM Daidzein) were used for a second investigation asking whether the demonstrated effect was a toxic one. Three further groups of aortic sections ("survival" or "28-days" groups) were treated with these concentrations over 21 days and then held in medium without 17β-estradiol/phytoestrogens for another 7 days. One group with 12 denuded aortic sections served as a 28 days control. Compared with the groups having been treated with 17β-estradiol/phytoestrogens in the same concentrations over 21 days, the 28 days survival groups showed a significantly increased neointima development, comparable to that of the control group held in medium with 1% isopropanol and 1% DMSO for 21 days (Fig. 2 and 3). The effect of post-treatment neointima formation was most in the Genistein treated group. Neointimaformation was significantly increased in the 28 days control group compared to the 21 days control group.

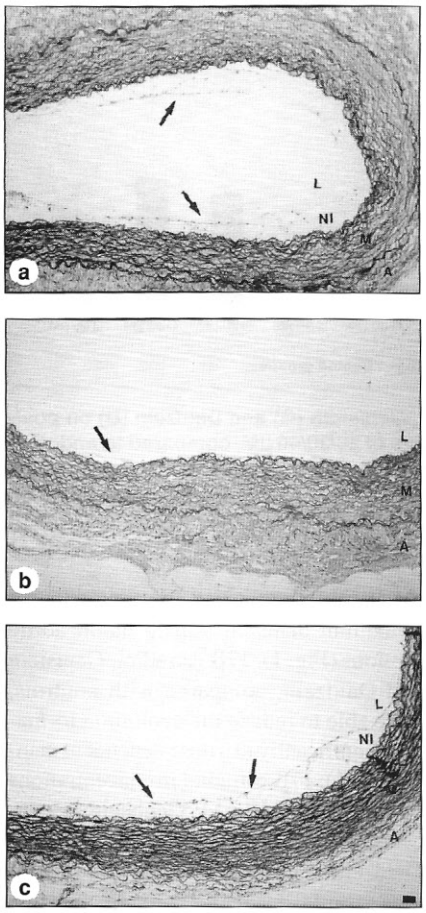

Figure 2 a-c: a) Neointima formation in a female rabbit aortic section after endothelial balloon denudation and 21 days of cultivation in medium containing 1% isopropanol and 1% DMSO. b) Inhibitory effect of 20 µM Genistein treatment over 21 days on neointima formation. c) Neointima formation after stopping 20 µM Genistein treatment and cultivation for another 7 days. Elastica-van-Gieson's staining. Magnification: balk is 10 µm. L = lumen; NI = neointima; M = media; A = adventitia.

3.2 Neointimal cell count

Compared with the control group those 17β-estradiol/phytoestrogen concentrations having been found to decrease neointima formation significantly over 21 days (30 µM 17β-estradiol, 20 µM Genistein, 40 µM Daidzein) were able to decrease the number of neointimal cells significantly as well (Fig. 4). However, in the Daidzein 28-days survival group the number of neointimal cells was significantly decreased compared to the 40 µM Daidzein (21 days) treated group (Fig. 4), indicating that the significantly increased amount of neointima formation in the 28-days group (Fig. 2) cannot be explained by the number of neointimal

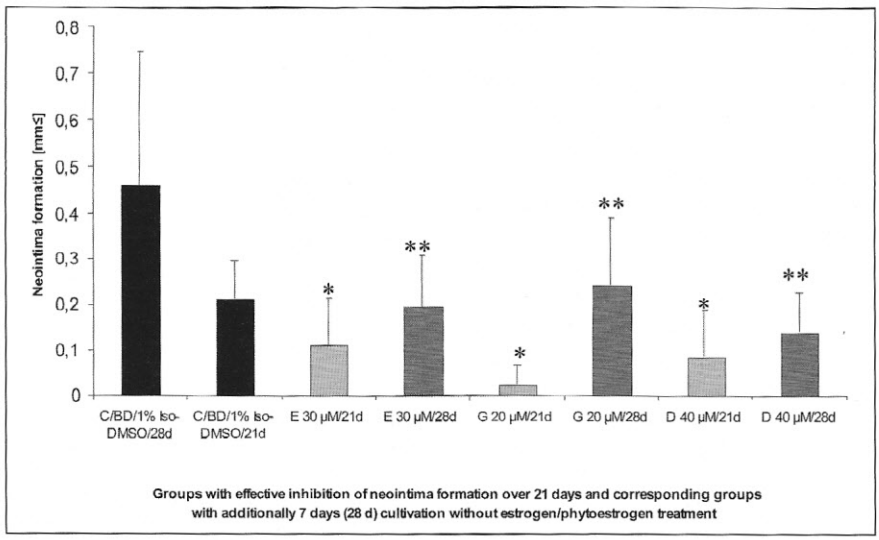

Figure 3: The lowest concentrations of 17β-estradiol (E), Genistein (G) and Daidzein (D) having been found to inhibit neointima formation significantly (*) after vascular injury over 21 days were used for a second experiment with treatment over 21 days. After this time the aortic sections were held in culture without 17β-estradiol/phytoestrogen treatment for another 7 days (28d) resulting in a significantly increased ($p<0.05$) neointima formation (**) compared to 21 days treated groups and comparable to the neointima formation of controls (C) held in medium containing 1% isopropanol (1% Iso) and 1% DMSO for 21 days.

cells. In the 30 µM 17β-estradiol treated group there was a discrepancy between the number of neointimal cells and post-treatment neointima formation as well (Fig. 2 and 4).

3.3 Proliferation

Compared with controls, 30 µM 17β-estradiol treatment over 21 days increased neointimal proliferative activity significantly while in the post-treatment stage (28-days survival group) the proliferative activity in the neointima was comparable to controls (Fig. 5). 20 µM Genistein treatment did not influence the proliferative activity in the neointima compared with controls, but in the post-treatment stage the proliferative activity increased significantly (Fig. 5). No significant changes were seen in the 40 µM Daidzein treated groups (21 days/28 days), however, there was a discrepancy between neointimal cell count and proliferative activity in the 28-days survival group (Fig. 4 and 5)

3.4 Staining for α-actin

The amount of α-actin positive cells in the neointima of the control sections was 51%-

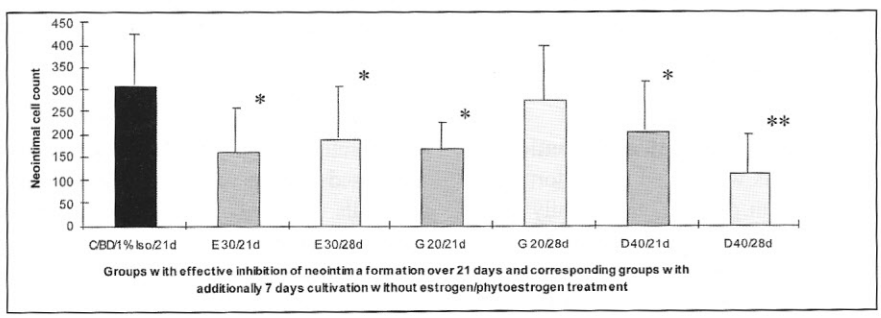

Figure 4: Corresponding to their ability to inhibit neointima formation after vascular injury, the lowest concentrations of 17β-estradiol (E), Genistein (G) and Daidzein (D) having been found to be significantly effective ($p<0.05$) were able to reduce the number of neointimal cells significantly (*) as well over 21 days of treatment. 17β-estradiol treated aortic sections, after another 7 days without 17β-estradiol application (28d), had still a significantly decreased number of neointimal cells compared to controls (C) held in medium containing 1% isopropanol (1% Iso) and 1% DMSO for 21 days. In Daidzein treated aortic sections (**) the number of neointimal cells once again decreased significantly ($p<0.05$) after the stop of treatment over 7 days (28d).

70% on an average, comparable to findings in all 17β-estradiol and Genistein treated groups (20 µM, 30 µM, 40 µM over 21 days). In all Daidzein treated groups the average amount of neointimal α-actin positive cells was 0%-25%. In the 28-days survival groups (post 17β-estradiol/phytoestrogen treatment) the amount of positive cells was 51%-100% on an average (Table 1).

4 Discussion

The purpose of the present study was to establish an *ex vivo* organ culture model for the investigation of post-injury estrogen effects in the vasculature and to proof whether these effects are caused just by toxicity. In this experiment, treatment with 30 µM 17β-estradiol, 20µM Genistein, or 40 µM Daidzein reduced neointima formation significantly over 21 days after injury. This effect was obviously not caused by cytotoxic mechanisms because, after the treatment had been stopped, these aortic sections were still able to proliferate and to build up a neointima.

Proliferation and migration of vascular smooth muscle cells (Ross, 1985; Rosenfeld and Ross, 1990) as well as the transformation of adventitial fibroblasts to neointima-invading and proliferating myofibroblasts (Shi et al., 1996) are key events in atherogenesis and restenosis.

Neointimal cells in this experiment were positive for α-actin staining and therefore by origin typical for medial vascular smooth muscle cells and adventitial myofibroblasts. Both are able to migrate to the neointima and to produce surrounding connective tissue (Layman and Titus, 1975; Wight and Ross, 1975; Shi et al., 1996). Moreover, α-actin staining serves as a marker for the integrity of tissue.

As a phenomenon, 17β-estradiol reduced neointima formation significantly at a concentration of 30 µM, but caused also a significantly increased proliferation at the same time indicating that its inhibitory effect on neointima formation cannot be explained by anti-proliferative but rather by anti-migrative properties and a suppression of matrix formation. While 20 µM Genistein and 40 µM Daidzein treatment did not reduce neointimal cell count, the explanation for their inhibitory effects on neointima development may be the suppression of matrix formation. Genistein in equivalent concentrations to 17β-estradiol and Daidzein had the strongest inhibitory effect on neointima formation. Stopping treatment resulted in a statistically significant increase of proliferative activity (BrdU staining) and in an increase (only by tendency) of neointimal cell count and neointima formation after one week of cultivation in medium only. However, the 28 days control group as well as the 28 days "survival" groups were incubated with 20% fetal calf serum (fcs) for the last 7 days. This

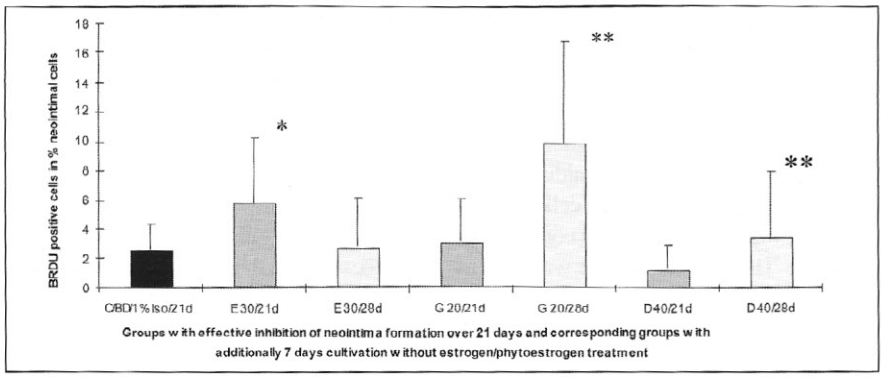

Figure 5: The proliferative activity in the neointima (BRDU positive cells in % neointimal cells) increased significantly (p<0.05) under 30 µM 17β-estradiol treatment over 21 days, compared to controls (C) held in medium containing 1% isopropanol (1% Iso) and 1% DMSO for 21 days. The stop of 20 µM Genistein and 40 µM Daidzein treatment (**) for 21 days resulted in a significantly (p<0.05) increased proliferative activity in the neointima after 7 days, compared to controls (C) and to 21 days treated groups.

Table 1: α-Actin positive neointimal cells

Score	+	++	+++	++++	n
Groups					
Control 21 days	0	1	**8**	**2**	11
17ß-estradiol 21 days					
20 µM	1	3	**5**	1	10
30 µM	3	3	**4**	1	11
40 µM	2	3	**6**	0	11
Genistein 21 days					
20 µM	0	0	**5**	**5**	10
30 µM	1	4	**5**	0	10
40 µM	1	2	**4**	**5**	12
Daidzein 21 days					
20 µM	**6**	**2**	2	0	10
30 µM	**4**	**2**	3	1	10
40 µM	**7**	**4**	0	0	11
17ß-estradiol 28 days					
30 µM	0	1	**5**	**5**	11
Genistein 28 days					
20 µM	0	0	**1**	**10**	11
Daidzein 28 days					
40 µM	1	3	**3**	**4**	11

Staining for α-actin was evaluated in α semi-quantitative manor in the neointima after 21 days of estrogen/phytoestrogen treatment and in aortic rings after 7 days without estrogen/phytoestrogen application. Score: + = 0–25% positive cells; ++ = 26–50% positive cells; +++ = 51–75% positive cells; ++++ = 76–100% positive cells. Controls, 17β-estradiol and Genistein treated aortic sections had 51–75% α-actin positive neointimal cells on an average. Daidzein treated rings had less α-actin positive neointimal cells (0–50%). After 7 days without estrogen/phytoestrogen treatment the average number of α-actin positive neointimal cells was increased to 51–100%.

resulted in an increase of neointima formation which may be caused by a number of growth factors in the fcs.

Vargas et al. (1993) previously described that 17β-estradiol in concentrations of 180-360 nM decreased the ^{3}H-thymidine uptake by vascular smooth muscle cells in segments of male and female pig coronary artery. The ^{3}H-thymidine uptake was used as a marker for DNA production and the resulting proliferation. Later on Chen et al. (1996) reduced myointimal proliferation in balloon injured carotid arteries of both female and male rats by inducing 17β-estradiol serum levels naturally found in female rats. So far, the here presented datas support these findings on estrogen's inhibitory effects on post injury processes in another model.

As several authors detected functional estrogen receptors in the arterial vasculature of animals (Lin et al., 1986; Venkow et al.; 1996) and humans (Karas et al., 1994; Losordo et al., 1994; Venkov et al., 1996), the vasoprotective effects of estrogen were supposed to be mediated possibly by estrogen receptor dependent pathways. Two different subtypes of the estrogen receptor, "ER-α" and "ER-β", were classified quite recently. Their activation leads to different, in part opposite estrogenic effects and may explain such paradox phenomena as estrogen having proliferative effects in one (i.e. endometrium) and antiproliferative effects in the other (i.e. vascular smooth muscle cells) tissue. Paech et al. (1997) could demonstrate *in vitro* that estrogen activated transcription with the ER-α but inhibited transcription with the ER-β, indicating that ER-β may be responsible for estrogens antiproliferative effects. Iafrati et al. (1997) supported this finding with mice *in vivo* by knocking out the gene coding for ER-α, resulting in antiproliferative effects of 17β-estradiol supposed to be mediated by ER-β. Mäkelä et al. (1999) demonstrated that after endothelial denudation of the rat carotid artery the mRNA of ER-α was expressed at a low level while ER-β mRNA increased more than 40 fold: to provide the suitable receptor subtype for 17β-estradiol to develop its antiproliferative properties. These findings still have to be reproduced in the rabbit aortic vessel.

The estrogenic power of phytoestrogens compared to endogenous 17β-estradiol varies target organ dependently. The uterotropic activity of Genistein in sheep is described to be not more than 1/1000th of that of 17β-estradiol (Lindner, 1976). Interestingly, Mäkelä et al. (1999) found that Genistein's affinity to ER-β is 20 fold higher than to ER-α, which would explain the comparably strong inhibitory effect on neointima formation in our present experiment. Whether the beneficial effects of 17β-estradiol or phytoestrogens in the vasculature are sufficiently explained by receptor mediated pathways requires further investigation. However, from experiments with human aortic smooth muscle cells (Dubey et al., 1999) we could learn that Genistein and Daidzein inhibited mitogen-induced proliferation, migration and extracellular matrix synthesis and that this effect could be blocked partially by pure estrogen receptor antagonists.

Fukumoto and co-workers (1996) suppressed restenosis after balloon injury in pig coronary arteries *in vivo* by use of an artificial tyrosine kinase inhibitor. It is well known that Genistein has tyrosine kinase inhibiting potencies (Akiyama et al., 1987) but that Daidzein has not (Jonas et al., 1995; Bischof et al., 1995) which would require another explanation for its antiproliferative effects. In this regard Jiang et al. (1991, 1992) found that estrogen has calcium antagonistic properties in vascular smooth muscle cells and that this could contribute to its beneficial post injury or antiatherogenic potencies. Calcium antagonistic effects are documented for Genistein as well (Chiang et al., 1996), but Daidzein has not yet been investigated in this regard. However, Genistein is under investigation focussing on its beneficial properties in tumor growth and angiogenesis, and there are more experiments indicating that Genistein is able to influence growth factor mediated pathways directly (Wei et al., 1995; Raines and Ross, 1995; Fotsis et al., 1995).

Phytoestrogens, some of them belong to the family of isoflavones, are natural components of nutrition such as soy beans (Coward et. al., 1996; Reinli and Block, 1996). High plasma concentrations can be measured in people consuming such food. This is thought to be responsible for the low mortality from prostatic cancer in Japanese men (Adlercreutz et al., 1993).

Since Anthony et al. (1996) have demonstrated beneficial effects of an isoflavone-enriched soy diet on plasma cholesterol levels in both female and male rhesus monkeys, isoflavones reached a special interest regarding their cardioprotective properties (Anthony et al., 1998). Recently, Honoré et al. (1997) demonstrated angiographically that Genistein injection caused acute vasodilation of atherosclerotic coronary arteries in primates. Further experiments will be necessary to elucidate how far antiproliferative properties of isoflavones may contribute to their clinical cardiovascular benefit. As shown above, phytoestrogens have only minimal effects on the reproductive system and may therefore offer a therapeutic option for men as well.

This *ex vivo* organ culture model will help to investigate distinct effects of ovarian or phytoestrogens and of pharmaca in general which are used in cardiovascular therapy (i.e., angiotensin converting enzyme inhibitors, AT_1-receptor antagonists, calcium antagonists), focussing on their potential effects on post-injury processes in the vessel wall. The rabbit model is widely accepted as a model for atherosclerosis research since first introduced by Anitschkow (1913), (Finking et Hanke, 1997). It is prior to some other *in vivo* models (i.e., dogs, pigs) because of a high quantity of available data which guarantees a good comparability. It is in some regards prior to the rat model because rats are resistant to dietary induced hypercholesterolemia which, on the other hand, can be combined quite successfully with vascular injury procedures in rabbits (Finking et al., 2000). A post-injury organ culture model with rabbit aortic vessels will offer frequent opportunities to investigate questions of physiological relevance under the primate to avoid the high number of animal experiments that otherwise would be necessary. Only findings of special interest may be investigated in an *in vivo* experiment later on. In this regard the organ culture model offers opportunities to investigate specific interactions, i.e., receptor mediated pathways by using specific receptor antagonists, enzyme or ion channel mediated pathways by using specific blockers or ion measurement techniques. Moreover this organ culture model offers the opportunity to test whether the demonstrated beneficial pharmacological effects have been caused just by cytotoxicity. Besides staining for proliferative activity (BrdU), α-actin, or active cellular transport (trypan blue), the "survival-test" will give one additional information on the tissue's integrity. Compared with a human arterial organ culture model (renal

arteries from nephrectomy patients) which has been established in our department quite recently (Voisard et al., 1999) this *ex vivo* model is distinguished by greater availability, homogenicity (sex of donor, age of vessel, preexisting disturbences, i.e., atherosclerotic plaques), and comparability. Because 24 aortic segments (rings) could be taken from one aortic vessel, the number of animals that would have been necessary for an *in vivo* experiment (8–10 for each group because of statistical reasons) could be markedly reduced (relation 1:16).

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