

Early-stage morphological observations of myoma and myometrium after laparoscopic uterine artery occlusion treatment

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ABSTRACT

Objective: Myoma therapy by uterine artery occlusion using laparoscopic ligation (UAOL) has been performed for many years and has proven effective, but limited information is available on its therapeutic mechanism. To examine this issue, we conducted this study to investigate the morphological change and apoptosis occurring in myomal and adjacent myometrial tissues shortly after UAOL.

Study design: In total, 16 myomas and adjacent myometrium were obtained from 7 cases before and at various points after artery ligation. The tissues were stained using hematoxylin and eosin for morphological observation. To investigate the existence of apoptosis, in situ immunostaining of Caspase 3 and TUNEL assay were performed. Cytochrome C released from mitochondria was also detected by immunohistochemistry.

Results: Microscopic observation found that after UAOL, both myometrial and myomal tissues were edematous and apoptotic cells were widespread in both tissues. TUNEL assays showed that before UAOL, numbers of apoptotic cells in myomal and myometrial tissues had no significant differences ($P = 0.866$). After ischemia of (36.69 ± 18.53) min, apoptosis was significantly more elevated in myoma than in myometrium $((6.43 \pm 4.38)/10$ HPF vs. $(2.74 \pm 1.95)/10$ HPF, $P = 0.003$). Caspase 3 stain shared similar features with the TUNEL assay. In both groups cytochrome C was released from mitochondria after UAOL, and more was detected in the myoma.

Conclusion: UAOL is an alternative method to treat symptomatic uterine myomas. Apoptosis via mitochondrial pathways may lead to reduction of the volume of myoma and myometrium and eventual relief of symptoms.

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1. Introduction

Uterine myoma is the most common benign tumor in the female genital tract, clinically present in about 25% of women. With the development of imaging techniques, the real clinical prevalence may even be higher [1]. Until recently, hysterectomy and myomectomy performed by either laparotomy or laparoscopy have been the primary treatment for symptomatic myomas.

Recently, a minimally invasive procedure, uterine artery occlusion (UAO), has been developed as an alternative to treat symptomatic myoma. The process can be achieved either by embolization (UAOE) or by laparoscopic ligation (UAOL). Both have been considered effective and safe to relieve symptoms of myoma [2–8]. Although there is widespread acceptance of UAO, the exact therapeutic mechanism has not been clearly identified. In 2000,

Burbank and colleagues put forward the “transient uterine ischemia” hypothesis to explain the mechanism of UAO. They proposed that after UAO, both myometrial and myomal vessels were occluded by clotting, resulting in organ ischemia [3]. Work on UAOL found that necrosis in the myoma, but not in the myometrium, contributed to the shrinkage of tumors, which was shown both through pathological observations and with Gd-enhancement on MR images [9,10].

Little was done on UAOL. According to Burbank's hypothesis, UAOL and UAOL share a common pathophysiologic process [3]. However, differences between these two procedures were obvious. Pathological exploration of UAOL samples often found embolic particles in the local vessels that frequently led to permanent damage of the vessel bed. But with UAOL, the vessels were intact and sometimes underwent later reperfusion [11]. The clinical courses of UAOL often showed severe pain and, less often, infection following this procedure [12]. Hysterectomy due to persistent pain or infection caused by uterine necrosis has been reported [13]. In contrast, to the best of our knowledge, no uterine necrosis in UAOL

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has been reported. Our recent large retrospective study on UAOL combined with myomectomy has demonstrated that the post-operative febrile morbidity was only 6%. In a 26-month follow-up, menstrual bleeding improved in 97%, and the uterine volume was reduced in 49% of the cases. The recurrence rate was 3%, and no symptomatic uterine necrosis occurred [8].

Park et al. conducted a study on the biopsy tissue of one patient 6 months after UAOL and found apoptotic cells in the myoma [14]. However, the period between UAOL and the biopsy was too long to explain the relatively short process of ischemia–reperfusion. We conducted this study to investigate the early morphological change of myomal and myometrial tissues after UAOL to provide concrete evidence for the therapeutic mechanism of UAOL.

2. Materials and methods

One hundred and fifty-three patients with uterine myoma were treated by a procedure of UAOL combined with later myomectomy in our hospital from January 2007 to January 2008. Seven patients from November 2007 to January 2008 were included in this study; all had been provided written informed consent before enrolment. The study was done in accordance with good clinical practice and Declaration of Helsinki guidelines. Documented approval from the ethics committee of our hospital was obtained. Those patients suffering from symptomatic fibroids and desiring to maintain an integral uterus or fertility in the future but not the near future were enlisted. Patients who had been taking hormonal therapy in the past 6 months or who had been diagnosed with adenomyosis or adenomyoma were excluded from this study. The major complaints of the patients included excessive bleeding and pelvic pain. The ages ranged from 38 to 47 years (43.29 ± 3.98 years). Sonography was performed to evaluate the size, number, and location of the myomas before a laparoscopic procedure was considered. In total, 16 intramural myomas were obtained from 7 cases: 3 cases with single myoma, 1 case with 2 myomas, 2 cases with 3 myomas, and 1 case with 5 myomas. The largest diameters of the myomas ranged from 20 to 73 mm (45.19 ± 14.45 mm). All samples of myoma and myometrium were identified by experienced pathologists before the experiments.

The procedure of UAOL and myomectomy was described in our previous article [8]. Briefly, the uterine arteries were isolated and occluded with PlasmaKinetic™ Forceps (Gyrus International, UK) under the guidance of a laparoscope. After a certain period of time, myomal pseudocapsules were dissected and myomas were stripped out. Before artery ligation, one myoma was removed from each patient. After that, myomas were removed one by one, and the time from blocking arteries to removing a myoma was recorded as ischemia duration. In this study, the ischemia durations of specimens were 10–75 min (36.69 ± 18.53 min). We also obtained synchronously a small sample of normal smooth muscle tissue adjacent to each myoma as control. All specimens were sent to the pathologist immediately. Half of each was cut into 4 μ m thick sections by freezing microtome and these were preserved at -20°C for later TUNEL assay. The other half was fixed in 10% formalin to prepare paraffin-embedded sections for hematoxylin and eosin and immunohistochemistry stains.

The 4- μ m paraffin-embedded sections of myomal and myometrial tissues for immunohistochemistry stain were deparaffinized in xylene, rehydrated in graded ethyl alcohol (100, 95, 80, 70%, then water), hot-treated in citric acid buffer at 99°C for 30 min to retrieve the antigens, and incubated in 3% hydrogen peroxide for 5 min to block endogenous peroxidase activity. The primary antibodies used were cytochrome C (Thermo Fisher Scientific Inc., USA) and Caspase 3 (Thermo Fisher Scientific Inc., USA). After the sections were incubated in a primary antibody solution overnight at 4°C and washed twice in a phosphate-

buffered saline (PBS) solution, they were incubated in biotin–immunoglobulin G antibodies (Thermo Fisher Scientific Inc., USA) for 30 min and then in streptavidin peroxidase (Invitrogen Corporation, USA) for 30 min. The chromogen detection system used was a DAB kit (Sigma Diagnostics, USA). The primary antibodies were replaced by PBS for negative control. Evaluation of Caspase 3 expression was performed as follows: each section was photographed at five random high-power fields ($400\times$); image capture and analysis were done using Nikon NIS-Elements AR software (Nikon Corporation, Japan). Cells showing a yellow or brown grainy stain were considered positive. Positivity was graded as 0+ (0–10%); 1+ (11–25%); 2+ (26–75%); and 3+ ($>76\%$). Each section was photographed at five random $1000\times$ fields for cytochrome C expression evaluation. Cells stained granularly with the nuclear region free from dye were considered negative. If the cytochrome C was released from mitochondria, the cells were stained diffusely and the nuclear region was covered and vague [15]. The number of positive cells per one $1000\times$ field was recorded for further analysis.

TUNEL analysis was performed using APO-BRDU-IHC kit (CHEMICON International Inc., USA) according to the manufacturer's protocol for staining of cryosections. The slides were stained by DAB and counterstained by methyl green (included in kit). A control slide offered by the manufacturer was simultaneously stained to confirm the results. Apoptosis was analyzed in a blind fashion without knowledge of the experimental group. The prepared slides were observed by microscope with a $40\times$ object lens for no fewer than 10 fields each to count the positive nuclei that shrank into varying sizes and were stained deep brown. The numbers of the positive cells per 10 high power fields (/10 HPFs) were recorded.

SPSS software (Version 11) was used for data analysis. The data were expressed as the mean \pm S.D. form. Non-parametric tests were performed to compare the differences between pre- and post-UAOL (two-sample Kolmogorov–Smirnov test) and between two kinds of tissues (Wilcoxon signed ranks test). The apoptotic cells detected by TUNEL and the positive cells of cytochrome C stain at different time points were linearly regressed against time series, and the regression coefficient (R^2) was presented.

3. Results

In myometrial tissues, smooth muscle cells (SMCs) were arranged in parallel to form bundles. Bundles were woven together with cell-scant connective tissue areas between them, where thick walled vessels and lymph ducts could be seen. The cavities of vessels were mostly flat. After the arteries blocked due to edema, cells and their nuclei became twisted and dilated, and the cell membrane was faint. Sporadic shrunk cells with dark nuclei and scant red cytoplasm could be seen, which were thought to be in apoptotic processes. Myomal tissues were relatively rich in smooth muscle cells (SMCs); they also formed a bundled structure, but lack of order. Unlike normal tissues, there were no cell-scant areas between bundles. The vessels in tissues were relatively small and there was a lack of smooth muscle layer. The spaces between SMCs were filled with thick collagen. After the arteries were blocked, edematous phenomena also existed, and small shrunk cells with dark nuclei spread sporadically (Fig. 1).

Results of Caspase 3 expression were shown in Table 1. This protein was expressed in cytoplasm of SMCs. Before UAOL, there was no significant difference between myomal and myometrial tissues ($P = 0.564$). After the UAOL, the positive cells were more numerous in myomal than in myometrial tissues and the difference was of statistical significance ($P = 0.001$). Compared with the expression of myomal tissues before UAOL, the positivity of post-UAOL elevated significantly ($P = 0.004$), but not the tissues of myometrium ($P = 0.500$).

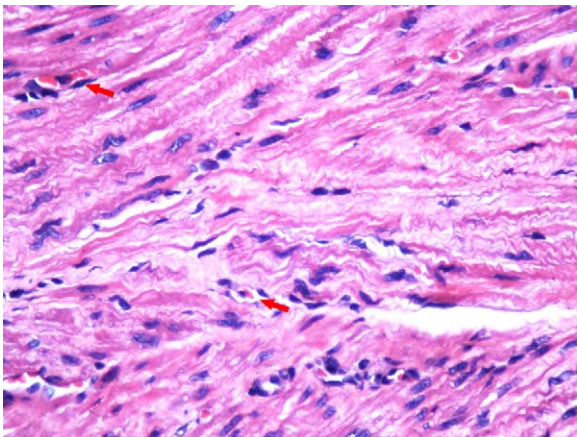


Fig. 1. H&E stain of myomal tissue after uterine artery occlusion by laparoscopic ligation. Smooth muscle cells were twisted, and two cells shrunk like apoptotic cells are arrowed (400 \times).

TUNEL assay showed that the positive nuclei (apoptotic cells) were stained deep brown and shrunk into dense masses of various sizes and shapes. The number of the positive cells per 10 high power fields (/10 HPF) was recorded. In pre-UAOL tissues, there was no difference between the myomas and the myometriums in the number of positive cells found ($(1.90 \pm 1.29)/10$ HPF vs. $(1.87 \pm 0.95)/10$ HPF, $P = 0.866$). After UAOL, with a mean ischemic duration of (36.69 ± 18.53) min, the positive cells increased more in the myomal than in myometrial tissues; there was a significant difference between them ($(6.43 \pm 4.38)/10$ HPF vs. $(2.74 \pm 1.95)/10$ HPF, $P = 0.003$). Furthermore, the positive cells were increased significantly more in myomas of post-UAOL than in pre-UAOL specimens ($P = 0.008$), while there was no difference between myometriums of pre- and post-UAOL ($P = 0.629$) (Table 2).

By the regression analysis of TUNEL assay, with the ischemia duration delaying, the positive cells of myomal but not myometrial tissues increased significantly. The parameters of time variable of myoma and myometrium were 0.153 ± 0.022 ($P < 0.01$) and 0.024 ± 0.015 ($P = 0.14$), respectively. The regression coefficients (R^2) were 0.698 and 0.100, respectively (Fig. 2). It showed a time dependent occurrence of apoptosis in the myomal tissues.

Table 1
Expression of Caspase 3 protein in paired myomal and myometrial tissue^a.

	Positive expression in cells ^b				P value ^c
	0+	1+	2+	3+	
Pre-UAOL ^d	3/3	3/4	1/0	0/0	0.564
Post-UAOL ^d	0/4	1/6	5/5	10/1	0.001

^a Data listed in the table are in the form of myomal/myometrial positive cells.

^b Positivity was graded as 0+ (0–10%); 1+ (11–25%); 2+ (26–75%); and 3+ (>76%).

^c By the Wilcoxon signed ranks test.

^d Myomal and myometrial tissues were distinctly compared with pre- and post-UAOL groups by the two-sample Kolmogorov–Smirnov test. The P values of myoma and myometrium were 0.004 and 0.500, respectively.

Table 2
Apoptosis in myomal and myometrial tissues pre- and post-UAOL detected by TUNEL assay.

	Numbers of specimen	Myoma	Myometrium
Pre-UAOL	7 pairs	1.90 ± 1.29 (0.63–3.85) ^{a,c}	1.87 ± 0.95 (0.91–3.33) ^{a,d}
Post-UAOL	16 pairs	6.43 ± 4.38 (1.33–16.67) ^{b,c}	2.74 ± 1.95 (0.00–6.67) ^{b,d}

^a Wilcoxon signed ranks test, $P = 0.866$.

^b Wilcoxon signed ranks test, $P = 0.003$.

^c Two-sample Kolmogorov–Smirnov test, $P = 0.629$.

^d Two-sample Kolmogorov–Smirnov test, $P = 0.008$.

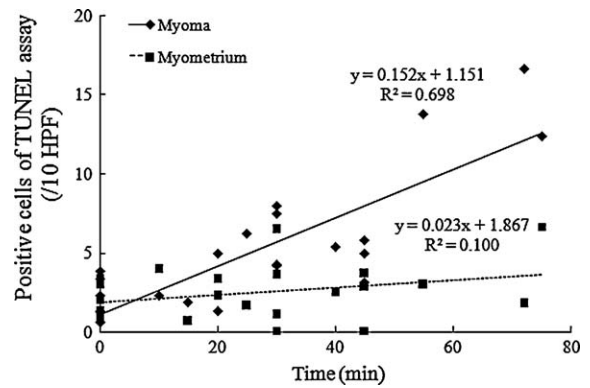


Fig. 2. Regression analysis of cytochrome C expression in myomal and myometrial tissues. The regression lines, formulae and the R^2 values are also given.

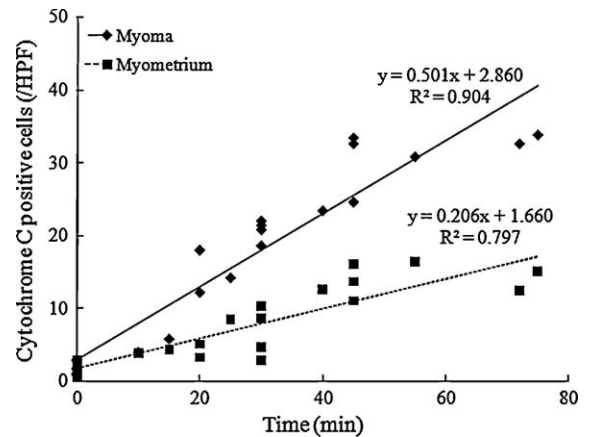


Fig. 3. TUNEL results were regressed according to the time series. The regression lines, formulae and the R^2 values are also given.

Different patterns in immunohistochemistry stain of cytochrome C were shown between myomal and myometrial tissues. Before UAOL, the cells of both groups showed granular positive stain, and the regions of nuclei were free from yellow stain. After UAOL, a part of the cells were stained diffusely, with a faint nuclear region, which represents the release of cytochrome C protein from mitochondria into cytoplasm. It was shown that with the delaying of ischemia, positive cells in both myomal and myometrial tissues increased. However, more were found in myomal tissues. Linear regression analysis showed that the coefficients of time parameter were 0.502 ± 0.036 ($P < 0.01$) and 0.206 ± 0.023 ($P < 0.01$), respectively. The R^2 were 0.904 and 0.797, respectively (Fig. 3).

4. Discussion

In recent years, uterine artery occlusion has gained popularity as an alternative to treat symptomatic uterine myomas. It can effectively reduce the volume of the myomas and the uterus, improving the symptoms of menorrhagia and bulk [2]. Two approaches, either by embolization or ligation, can be chosen to perform this procedure. However, its pathophysiological progress and underlying mechanisms are less known. Burbank et al. proposed that the ischemia–reperfusion process led to the death of myomas by both methods [3]. With limited pathological information, primarily in UAOL after failed operation or uncontrollable post-operative complications, necrosis was found in myomas and was considered to contribute to the therapeutic effect. Embolic particles found in the local vessels reinforced the theory of insufficient reperfusion of the myomas [9,16].

However, infarction, with loss of all blood flow to myomas, is not necessary when myomas are treated with UAOL. Lee et al. performed color Doppler sonography to detect the blood flow in myoma, and found less flow 1 week and 4 months after UAOL accompanied the relief of symptoms [17]. Park et al. studied UAOL in 23 cases and UAOL in 17 cases, and found that both methods were effective, but necrosis was only noted in UAOL cases, with only one sample of the myoma 6 months after UAOL, in which the author found apoptosis [14].

By performing UAOL combined with later myomectomy, we had the opportunity to get much closer to the mechanism of UAOL. The pathophysiological process simulated a shock process but occurred only within a single organ (i.e., uterus shock). We found the myomal and myometrial tissues were both edematous, and edema was not confined only to the myoma. With various approaches, we confirmed that apoptotic cells occurred in both types of tissues but more were seen in myomas. As the duration of ischemia increased, the positive cells increased significantly in myomal specimens and increased slightly in myometrium tissues. Our results provide direct evidence that apoptosis might cause the volume reduction of not only myomas, but also the uterus itself. It may be similar to the reduction of postpartum uterine volume [18].

Apoptosis and necrosis were originally described to be two types of cell death which can occur simultaneously in tissues exposed to ischemia–reperfusion. The intensity and duration of ischemia–reperfusion may determine the outcome [19]. Theoretically, occluded by polyvinyl alcohol particles, the myomal and/or myometrial vasculature was damaged completely during UAOL, resulting in non-reperfusion status and necrosis. Compared with UAOL, the vasculature of the uterus was intact during UAOL procedure, thus the possibility of reperfusion from communicative branch remained, resulting in a mild ischemia–reperfusion injury. However, different anatomic features of blood supply [20] and/or blood coagulation and fibrinolysis activities [21] between myoma and myometrium might also explain uterine survival and myomal death.

In this study, the apoptosis rates of both experimental and control groups were low. These results can probably be attributed to the fact that the apoptotic peak level was not reached during the observed time range of 75 min, confined by the surgery time. Further investigations are necessary to elucidate the biological differences between myoma and myometrium with cultured cells, and the difference of blood supply between myoma and myometrium. These approaches may be helpful in unraveling the mechanism of UAOL for treatment of myoma.

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